REMARKS/ARGUMENTS

With entry of this amendment, claims 2-21 are pending in the above-identified application. Claims 2, 8, and 12 are amended and new claims 20 and 21 are added as set forth in detail below. Support for these amendments are identified in the following remarks. No new matter has been added. Examination and reconsideration of all pending claims are respectfully requested.

Priority

The Examiner states that "the priority determination for the instant Application has been made only as far as the filing of PCT/JP00/06313 due to the absence of English translations of the foreign priority documents."

This issue was previously raised in the Office Action mailed February 12, 2004. This issue was also previously addressed in Applicants' response to the 2/14/05 Action. In particular, Applicants noted that, according to the MPEP, English translations of the priority documents may only be required by the Examiner when there is an intervening reference, which is not the case here. The MPEP states as follows:

> The only times during *ex parte* prosecution that the examiner considers the merits of an applicant's claim of priority is when a reference is found with an effective date between the date of the foreign filing and the date of filing in the United States and when an interference situation is under consideration....In those cases where the applicant files the foreign papers for the purpose of overcoming the effective date of a reference, a translation is required if the foreign papers are not in the English language.
>
> [MPEP § 201.15.]

In the present case, the three references cited by the Examiner predate the earliest priority document. Therefore, as previously noted in Applicants' response to the 2/14/05 Action, the Examiner need not make any comment on whether Applicants are entitled to the claimed priority date.

Claim rejections under 35 U.S.C. § 102

Vito *et al.*

Claims 2, 4-8 and 10-17 stand rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Vito *et al.* (IDS reference AG). This rejection is overcome in part and traversed in part as set forth hereinbelow.

The Examiner takes the general position that "the phrase 'obtained from or synthesized from a nucleic acid obtained from a tissue of an organism suffering from a disorder, wherein said tissue is obtained from an organ showing cell death as a pathological feature of the disorder' contains 'reach-through' language as it regards the source of the nucleic to be expressed in the cell." (Office Action dated March 25, 2005, at page 3.) The Examiner further states that, in order for such language to carry patentable weight, "there must be some structural or functional variation conveyed upon the nucleic acid as obtained from one source over another." (*Id.*) With particular regard to the Vito reference, and on the basis of the Examiner's interpretation summarized above, the Examiner goes on to contend that because the cDNA library of Vito is obtained from cells undergoing PCD, the nucleic acids of Vito are identical to those obtained from an organ undergoing cell death.

First, Applicants note that the Examiner has provided no legal basis to support the position that the recited source of a nucleic acid can be ignored in a process claim. As set forth in the MPEP, in assessing whether a reference anticipates a claim, each and every limitation as recited in the claim must be considered. *See* MPEP § 2131. While an exception to this rule has been made in the case of product-by-process claims (*see id.* at § 2113), such an exception has not been made by the courts with respect to "process-by-process" claims. Accordingly, with respect to the process claims currently pending, because the Examiner has not cited to any statute or case that supports the position that the source of the nucleic acid can be ignored, the nucleic acid source as recited in the claims must be considered in determining novelty. For at least this reason, Applicants believe the present claims to be novel over Vito.

While Applicants do not agree with the Examiner's interpretation of the pending claims as it relates to the cited references, but in order to expedite prosecution of this application, Applicants have amended independent claims 2 and 8. Claims 2 and 8 now specify that a library of nucleic acids, obtained from or synthesized from nucleic acids expressed in the tissue showing cell death as a pathological feature of the disorder, is expressed in a population of cells. Support for this amendment is found in the specification at, *e.g.*, page 3, line 18, bridging to page 4, line 5; page 5, lines 6-18; page 8, lines 14-24; and page 12, line 33, bridging to page 13, line 15.

In view of the above amendments, Applicants note that the present claims are directed, *inter alia*, to a library of nucleic acids derived from cells expressing genes during disease pathogenesis in an *in vivo* context. In contrast, Vito discloses the expression of a cDNA library constructed from mRNA of an *in vitro* cultured cell line artificially stimulated using an antibody specific for CD3ε. Vito used a single cell species, 3DO, which is an immortalized hybridoma formed by the fusion of a mouse T cell with a thymoma cell (*see* Ashwell *et al.*, *J. Exp. Med.* 165:173, 1987, at page 174, last paragraph (attached hereto as Exhibit 1), cited by Vito in item 3 of "References and Notes").

While any particular nucleic acid obtained from the cells of Vito may or may not be identical to a nucleic acid obtained from a cell of a tissue obtained from an organ undergoing cell death as a pathological feature of a disorder, a library of expressed nucleic acids as disclosed by Vito would not be identical to a library of expressed nucleic acids as recited in the present claims. It is well-known in the art that homogenous populations of cells cultured *in vitro*, especially immortalized cell lines, do not entirely replicate the physiological conditions or gene expression patterns of cells *in vivo*. Accordingly, the skilled artisan would readily understand that the *in vivo* gene expression patterns of a tissue obtained from a diseased organ would differ, both qualitatively and quantitatively, from gene expression patterns of *in vitro* cultured 3DO cells (*i.e.*; would display differences in the identity of genes expressed as well differences in levels of expression of certain genes). Thus, a library of nucleic acids obtained from or synthesized from nucleic acids expressed *in vivo* in a tissue obtained from a diseased organ would show structural variation (with respect to the frequency of expressed sequences as well as

identity of certain sequences) as compared to a library constructed from the immortalized 3DO cell line cultured *in vitro*.

With specific regard to the Examiner's point that that the nucleic acids of Vito were "obtained from cells undergoing PCD," Applicants further note that the conditions used in Vito for inducing apoptosis in 3DO cells are not substantially representative of conditions that would be encountered physiologically *in vivo*. As noted above, the 3DO cells of Vito were stimulated with anti-CD3ε 2C11 antibody (an antibody specific for a particular subunit of the T cell receptor). However, under physiological conditions *in vivo*, several other factors modulate apoptotic death of T lymphocytes, including, *e.g.*, interleukins, glucocorticoid hormones, and adhesion receptors. (*see, e.g.*, Ayroldi *et al.*, *Blood* 86:2672-2678, 1995, at page 2677 (Discussion), attached hereto as Exhibit 2). Some of these factors are capable of inducing specific pathways in lymphocytes that inhibit TcR-mediated apoptosis. (*See id.*) Cells showing such differences in activation of apoptotic and survival pathways would be expected to show differences in gene expression patterns. Accordingly, for these reasons in addition to the reasons above, Applicants respectfully submit that the cDNA library of Vito, constructed from a homogenous culture of 3DO cells stimulated using an anti-CD3 antibody but without the presence of other apoptosis-modulating factors found *in vivo*, is structurally and functionally distinguishable from the nucleic acid library recited in the present claims.

The presence of additional factors *in vivo* that modulate apoptosis is particularly relevant to the present invention. The present invention is directed to the use of a library of nucleic acids "condensed" or enriched for disease-suppressor genes. (*See* specification at, *e.g.*, page 3, line 18, bridging to page 4, line 5.) As set forth in the specification, the present inventors appreciated that, in disorders accompanying cell death, cell death does not always occur in all cells contained in the affected areas, and that tissues in the vicinity of the affected area may sufficiently express suppressor genes preventing the development of pathological symptoms. (*See* specification at page 3, line 14, to page 4, line 5.) By using such tissues to construct a nucleic acid library, a library condensed for disease-suppressors can be obtained. Expressing such a condensed library of disease-suppressor genes within a cell population allows for more efficient screening assays to identify the disease suppressor genes, based on the suppressive

effect of these genes on a disorder. (*See* specification at, *e.g.*, page 3, lines 14-21.) For substantially the same reasons as set forth above, the skilled artisan would not reasonably view *in vitro* cultured 3DO cells, artificially stimulated only through the TcR with an anti-CD3ε antibody, as containing physiologically relevant disease-suppressing factors to the same degree as a tissue actually obtained from an organ showing cell death as a pathological feature of a disorder.

For the reasons above, Applicants believe claims 2, 4-8, and 10-17 to be novel over Vito *et al.* Withdrawal of the rejection is respectfully requested.

Guo *et al.* and Giambarella *et al.*

Claims 2, 4-8, and 10-17 stand rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Guo (as cited in the previous Office Action). Claims 2, 4-8, and 10-17 also stand rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Giambarella *et al.* (as cited in the previous Office Action). These rejections are overcome in part and traversed in part as set forth below.

Applicants initially note that the Examiner has maintained these rejections on the basis of the interpretation of the phrase "obtained from or synthesized from a nucleic acid obtained from a tissue of an organism suffering from a disorder, wherein said tissue is obtained from an organ showing cell death as a pathological feature of the disorder" as containing "reach-through" language with respect to the source of the nucleic to be expressed in the cell. The Examiner states that the "nucleic acid being expressed is the function element of the claims [that] is relevant ... with regard to patentability." (Office Action dated March 25, 2005, at page 5.) The Examiner goes to state that, in both Guo and Giambarella, "it is clear that the nucleic acids exhibiting a suppressive effect on apoptosis can be obtained from an organ undergoing cell death, given the source of the suppressor nucleic acid and absent evidence to the contrary." (*Id.* at page 6.)

As noted above with respect to the Examiner's rejection in view of Vito *et al.*, the Examiner has provided no legal basis to support the position that the recited source of a nucleic

acid can be ignored in a process claim. Accordingly, the nucleic acid source as recited in the pending claims must be considered in determining novelty. For at least this reason, Applicants believe the present claims to be novel over each of Guo and Giambarella.

While not agreeing with the Examiner's rejections in view of Guo and Giambarella as set forth above, Applicants believe these rejections to be obviated in light of the claim amendments previously set forth with respect to the rejection over Vito *et al.* The claims as amended now specify that a library of nucleic acids, obtained from or synthesized from nucleic acids expressed in the tissue showing cell death as a pathological feature of the disorder, is expressed in a population of cells.

It is well-known that while diseased and corresponding normal cells typically comprise the same or substantially the same genome, the *in vivo* gene expression patterns in a diseased tissue differ from gene expression patterns of a corresponding normal tissue (*i.e.*, diseased tissue displays differences in the identity of genes expressed as well as differences in levels of expression with respect to certain genes). Accordingly, a library of nucleic acids obtained from or synthesized from nucleic acids expressed in a diseased tissue would show structural variation, *e.g.*, with respect to the identity of certain genes expressed as well as the frequency of specific sequences in the library, as compared to a library derived from normal tissue. In particular, and as noted above in response the Examiner's rejection in view of Vito *et al.*, by constructing a library of nucleic acids derived from nucleic acids expressed in a tissue obtained from an organ undergoing cell death as a pathological feature of a disease, a library "condensed" or enriched for disease-suppressor genes can be obtained. (*See* specification at, *e.g.*, page 3, line 14, to page 4, line 5.)

To further support the novelty of the claims as currently amended, particularly with regard to structural variation *vis a vis* a nucleic acid expression library obtained from the tissue of diseased and normal organs, Applicants have attached hereto Exhibit 3 (Tajima *et al.*, *Neuroscience Letters* 324:227-231, 2002). Tajima *et al.* shows the expression profile of Humanin ("HN," a neuroprotective polypeptide described in the Examples of the instant

specification) in an Alzheimer's disease (AD) brain. Specifically, Tajima *et al.* states the following:

> In an AD brain, HN immunoreactivity was detected in some of the intact large neurons in the occipital lobes (Fig. 3f). There was no similar immunostaining in neurons in an occipital lobe in an age-matched control brain (Fig. 3e). In the AD brain, HN immunoreactivity was also detected in small, round reactive glias (Fig. 3d, left panel). This type of immunoreactivity was widely distributed in the AD brain, most abundantly in the hippocampus. The age-matched control brain exhibited only few HN-immunoreactive glias (Fig. 3a).

(Tajima *et al.* at page 229, 2nd col., last paragraph, bridging to page 230, first column.) Thus, a nucleic acid expression library obtained from the tissue of a diseased organ is comprised of an expressed gene population structurally distinguishable from that obtained from normal tissue.

With regard to the Examiner's assertion that the nucleic acid is the functional element of the claims, Applicants note that such structural variation in a nucleic acid library would also impart functional variation when expressed in a population of cells. It is axiomatic that, when introduced into a cell population, the gene population represented in a particular expression library is reflected in a population of transfected cells displaying the functional characteristics associated with the products of the gene sequences in the library. Thus, which functional characteristics are present in a transfected cell population, as well as the frequency of transfected cells displaying a particular functional characteristic, will depend on the identity and frequency of sequences represented in the library.

This functional variation has certain technical advantages. For example, imparting a higher frequency of a particular functional characteristic to a cell population allows for more efficient screening of such cells based on the function. In the present case, expressing a condensed library of disease-suppressor genes within a cell population allows for efficient screening assays to identify a disease suppressor gene without necessarily having *a priori*

knowledge of the sequences being expressed, based on the suppressive effect of these genes on a disorder. (*See* specification at, *e.g.*, page 3, lines 14-21.)

In view of the above remarks and the present amendments to the claims, neither Guo nor Giambarella anticipate the present claims under 35 U.S.C. § 102. The claims, directed to a screening method, require that a library of nucleic acids be expressed in a population of cells. In each of Guo and Giambarella, a cell population is transfected with a single cDNA sequence, with the authors having *a priori* knowledge of the cDNA sequence being expressed. Guo discloses the expression of rat calbindin D28K cDNA in PC12 cells; while Giambarella discloses the expression of βARK cDNA in NK1/Puro cells. Neither reference discloses expressing a nucleic acid library in a cell population, much less a library of nucleic acids expressed in a tissue obtained from an organ undergoing cell death as a pathological feature of a disorder.

Accordingly, for at least the reasons above, Applicants believe claims 2, 4-8, and 10-17 to be novel over each of Guo *et al.* and Giambarella *et al.* Withdrawal of the rejections is respectfully requested.

Claim rejections under 35 U.S.C. § 103

Claims 2, 4-8, 10-17 and 18-19 stand rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Vito *et al.* in view of Slamon *et al.* (U.S. 6,770,477).

Applicants believe the present rejection to be obviated in view of the amendments and remarks set forth in response to the rejection in view of Vito *et al.* Because amended claims 2 and 8 are patentable over Vito *et al.* for the reasons above, all claims depending therefrom should also be patentable. Withdrawal of the rejection is respectfully requested.

Other Claim Amendments

Claim 12 has been amended to correct a typographical error. Claim 12 now recites "The method according to claim 11 …."

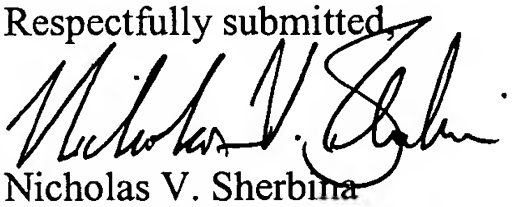
New dependent claims 20 and 21 have been added. Claims 20 and 21, which depend from claims 2 and 8, respectively, each recite an embodiments of the claimed method further comprising "obtaining the nucleic acids expressed in the tissue of the organism suffering from the disorder; and ... constructing the library of nucleic acids therefrom." Support for these claims is found in the specification at, *e.g.*, page 4, lines 1-5; page 12, lines 13-16; and page 12, lines 33, bridging to page 13, line 15.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 206-467-9600.

Respectfully submitted,



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CELL GROWTH CYCLE BLOCK OF T CELL HYBRIDOMAS UPON ACTIVATION WITH ANTIGEN

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For many years the study of cellular immunology was impeded by the inability to propagate and expand homogeneous populations of antigen-specific lymphocytes in vitro. In the mid-1970's, Köhler and Milstein (1, 2) developed a technique that allowed the immortalization of antigen-specific B cells by fusing them to transformed and spontaneously proliferating myeloma cells, thereby producing B cell hybridomas. Hybridoma technology was subsequently applied to T lymphocytes, providing a convenient source of T cells for in vitro study (3–5), and the establishment of antigen-specific T cell hybridomas made it possible to analyze the requirements for antigenic stimulation in model systems (6). Murine antigen-specific T cell hybridomas are commonly prepared by using polyethylene glycol to fuse antigen-primed T cell blasts to the spontaneously proliferating AKR-derived thymoma, BW5147. These T cell hybridomas can be tested for their ability to respond to the antigen used to prime the normal lymphocyte fusion partner, and the cells that exhibit the appropriate specificity can be cloned and expanded to large numbers.

For normal T lymphocytes, occupancy of the antigen receptor usually results in a complex series of activation events that include expression of new cell surface receptors, production of lymphokines, and entry of the cell into its growth cycle (7). This last response is not pertinent when using T cell hybridomas, because they are spontaneously proliferating cells. Therefore, the approach that has generally been used to assess the result of antigen receptor occupancy of T cell hybridomas is to quantitate their production of lymphokines, typically IL-2 (6). In recent years it has become evident that the occupancy of a variety of receptors that typically promote growth in normal cells, e.g., occupancy of receptors for epidermal growth factor (8), transforming growth factor β (9), and even IL-2 (10, 11), can inhibit the growth of certain transformed cells. We therefore undertook an analysis of the effect of antigen-mediated activation upon the growth of murine T cell hybridomas. We found that stimulation with antigen resulted in a dose-dependent decrease in both [^{3}H]thymidine incorporation and cell growth. This phenomenon displayed the same ligand-specificity as did IL-2 production, as demonstrated by its requirement for a specific allelic form of the Ia molecule, anticlonotypic (i.e., anti–antigen receptor) antibody blocking exper-

EXHIBIT 1

iments, and stimulation with anticlonotypic antibodies crosslinked to Sepharose beads. Flow cytometric analysis revealed that antigen activation produced a block in the cell growth cycle that was predominantly at the G_1/S interface, although cells also appeared to be slowed or halted in S phase. Furthermore, with the use of correlated DNA and surface immunofluorescence flow cytometry, it was demonstrated that the addition of antigen to a mixture of T cells specific for different antigens resulted in a cell cycle block in only the stimulated T cell hybrid. The implications of this phenomenon are discussed, including its utility as a rapid and quantitative measure of T cell hybridoma activation, as a means of selecting T cell hybridomas that have functional mutations in the antigen-specific receptor or elsewhere in the activation pathway, and as a possible model for the induction of T cell tolerance.

Materials and Methods

Animals. B10.A/SgSn (B10.A) mice were obtained from Harlan Spraque-Dawley (Madison, WI) through a contract with the Animal Genetics Production Branch (Developmental Therapeutics Program, Division of Cancer Treatment, NCI, Frederick, MD). B10.D2 mice were derived from pedigreed pairs originally obtained from Dr. Jack Stimpfling, Great Falls, MT. Mice of either sex were used between the ages of 2–12 mo.

Antigens. Pigeon cytochrome *c*, chicken ovalbumin, and hen egg lysozyme (HEL)[1] were purchased from Sigma Chemical Co. (St. Louis, MO). The random terpolymer poly-$(Glu^{60}\text{-}Ala^{30}\text{-}Tyr^{10})_n$ (GAT) was purchased from Vega Biochemicals (Tucson, AZ). The COOH-terminal pigeon cytochrome *c* fragment 81–104 was prepared by CNBr cleavage as described (12).

Antibodies. 10-2.16 is a murine IgG2b antibody that binds the A^k_β:A^k_α Ia molecule (13). 14.4.4 is a murine IgG2a antibody that recognizes the E^k_β:E^k_α Ia molecule (14). Both antibodies were prepared as ascites and used at a final dilution of 1:500 (vol/vol). A2B4-2 is a murine IgG2a antibody that binds the antigen-specific receptor borne by the T cell hybridoma 2B4 (15). Ascites containing A2B4-2 was purified by sequential passage over columns of DEAE Affi-Gel Blue (Bio-Rad, Richmond, CA) and protein A–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). The purified antibody was coupled to activated Sepharose (Pharmacia Fine Chemicals) at a concentration of 2 mg/ml (determined spectrophotometrically), and was the generous gift of Drs. H. Narimatsu and R. Schwartz (National Institute of Allergy and Infectious Diseases, NIH). For the blocking experiments, the anticlonotypic antibody A2B4-2 was purified from ascites in a single step by passage over a protein A–Sepharose column, and was kindly provided by Dr. B. Fox (National Institute of Allergy and Infectious Diseases, NIH).

T Cell Hybridomas. All of the T cell hybridomas used in this study were obtained by polyethylene glycol–mediated fusion of antigen-primed lymph node T cells to the AKR-derived thymoma BW5147 as described (16). The strain and haplotype of the BW5147 fusion partners, the antigen/Ia molecule specificity of the hybridomas, and a reference in which each hybridoma was described are given in Table I. The T cells 2B4.11 and C10.9 were obtained by subcloning the 2B4 and C10 lines, respectively, at limiting dilution, and were selected for use in these studies because of their stability as judged by their functional responses, and in the case of 2B4.11, the homogeneous expression of the antigen-specific receptor. The T cell hybridomas A4.A1.4 and A6.A2.7 were obtained by subcloning the A4.A1 and A6.A2 lines, respectively, at limiting dilution, and were selected for their superior ability to produce IL-2 compared to the parent cell line. All of the T cell hybridomas were maintained in exponential growth in a medium consisting of RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% heat-inactivated fetal calf serum,

[1] *Abbreviations used in this paper:* CTLL, IL-2-dependent cytotoxic T cell line; EGF, epidermal growth factor; HEL, hen egg lysozyme.

4 mM glutamine, 100 U/ml penicillin, 150 μg/ml gentamicin, and 50 μM 2-ME (growth medium).

Stimulation of T Cell Hybridomas. 5–10 × 10^3 T hybridoma cells were cultured in duplicate or triplicate in 96-well, flat-bottomed microtiter plates (3596; Costar, Cambridge, MA) in a final volume of a 50:50 (vol/vol) mixture of RPMI 1640 (Biofluids) and Eagle's-Hank's amino acid (EHAA) medium (MA Bioproducts, Walkersville, MD) supplemented as above (complete medium). In the case of antigen stimulation, 5 × 10^5 irradiated (3300 rad) splenocytes were added to each well along with the indicated concentrations of antigen. Stimulation with antibody-coupled beads was performed by resuspending a 50:50 (vol/vol) slurry of A2B4-2-coupled Sepharose beads and removing the volumes indicated. The beads were washed with PBS three times, resuspended in complete medium, and added to the T cells in the absence of any other cell type (i.e., no splenocytes were present).

IL-2 Assay. After 24 h of culture, 50 μl of supernatant was removed from each well and frozen to ensure that no viable cells were included. The IL-2 content was determined by incubating an IL-2-dependent T cell line (CTLL) at 3 × 10^5 cells/well with twofold serial dilutions of the supernatant. After ~20 h, the CTLL were pulsed with 1 μCi of [^{3}H]thymidine per well (6.7 Ci/mmol; ICN, Irvine, CA) and, after another 8 h, were precipitated onto glass filter strips with the use of a semiautomated cell harvester (PHD Cell Harvester; Cambridge Technology, Cambridge, MA). The incorporation of [^{3}H]-thymidine was determined by liquid scintillation counting. One unit of IL-2 activity is defined as that dilution of supernatant capable of stimulating the IL-2-dependent T cell to half of its maximal proliferative response.

[^{3}H]Thymidine Incorporation by T Cell Hybridomas. At 24 h of culture, after 50 μl of supernatant had been removed for the measurement of IL-2, 1 μCi of [^{3}H]thymidine was added to each T cell hybridoma-containing well (5–10 × 10^3 cells/well in 150 μl). 2–3 h later the wells were harvested as above, and [^{3}H]thymidine incorporation was assessed by liquid scintillation counting.

Cell Counting. 2B4.11 or C10.9 T cells were placed in 96-well flat-bottomed plates at a density of 5 × 10^3 cells/ml in complete medium. 5 × 10^5 irradiated (3,300 rad) B10.A (syngeneic), or B10.D2 (allogeneic), splenocytes were added to each well as a source of APC, along with varying amounts of the appropriate antigen. At 24-h intervals, three wells were resuspended by gentle pipetting, 10–20-μl aliquots were removed, and viable cells were counted in the presence of 0.4% trypan blue stain (Gibco Laboratories, Grand Island, NY) by light microscopy. T hybridoma cells were easily differentiated from the irradiated splenocytes on the basis of morphology and the exclusion of trypan blue dye. The standard error of these determinations was always <10%.

Flow Cytometry. T cell hybridomas were cultured at 4 × 10^5 cells/ml in 5-ml plastic tubes (Becton Dickinson, Oxnard, CA) containing 5 × 10^6 irradiated (3,300 rad) B10.A splenocytes in the presence or absence of antigen. After 24 h, viable cells were isolated by recovery from a Lympholyte-M density gradient (Cederlane Laboratories, Ontario, Canada), washed in saline G (PBS containing 1 g/liter glucose without any calcium or magnesium, prepared by the NIH media unit), and the cell pellet was fixed by slowly adding chilled absolute ethanol while vigorously mixing the cells. After fixing for 2–24 h in ethanol, the cells were centrifuged, washed in saline G, and resuspended in saline G containing 1 mg/ml of RNase A (Worthington Biochemicals, Freehold, NJ) and 18 μg/ml of propridium iodide (Sigma Chemical Co.). The cells were allowed to equilibrate for at least 1 h before being analyzed for cellular DNA content with a Cytofluorograf System 50 (Ortho Diagnostic Systems, Westwood, MA) using 488-nm excitation at 200 mW. Doublets and higher aggregates were excluded from analysis by using correlated area/peak measurements of DNA content similar to that described previously (21). Cell cycle analyses were done by the polynomial model of Dean and Jett (22). Cell size was simultaneously measured by using axial extinction at 633 nm from a 0.8 mW helium-neon laser. For correlated DNA/surface immunofluorescence measurements, ~1–2 × 10^6 2B4.11 or C10.9 cells (or a mixture of both) were incubated in HBSS (Biofluids) containing 0.2% NaN_3, 1% fetal calf serum; and with or without 100 μl of A2B4-2 culture supernatant (a

saturating concentration, data not shown) for 30 min at 4°C. The cells were washed three times and incubated with 5 μg of affinity-purified fluoresceinated goat anti-mouse Ig $F(ab')_2$ (Cappel Laboratories, West Chester, PA) for 45 min at 4°C. The cells were washed twice in saline G before fixation with ethanol. Analyses were performed using a Cytofluorograf System 50 at 200 mW laser power at 488 nm. Under these conditions, the red fluorescence associated with DNA could be easily separated from the green fluorescence associated with the cell surface A2B4-2 antigen by appropriate filtration. Contour plots were generated using software developed by Dr. Peter Rabinovitch (University of Washington) for the Ortho 2150 computer system.

Results

Stimulation with Antigen Inhibits Uptake of [^{3}H]Thymidine by T Cell Hybridomas. In the course of studies concerning the effect of varying T cell number upon antigen-stimulated IL-2 production, we noted that T cell hybridomas appeared to grow more slowly in the presence of their antigen. To quantitatively assess this phenomenon, a variety of antigen-specific T cell hybridomas were cocultured with antigen-presenting cells (APC) and assayed for their ability to incorporate [^{3}H]thymidine in the presence or absence of antigen (Table I). Eight T cell hybrids of varying antigen- and major histocompatibility complex (MHC)-specificity were tested; all displayed a marked inhibition of [^{3}H]thymidine incorporation 24–26 h after the addition of the appropriate antigen. In all cases, the T cell hybridomas were found to produce measurable amounts of IL-2 at concentrations of antigen that caused substantial inhibition of [^{3}H]thymidine incorporation. Two of the hybridomas, 2B4.11 and C10.9, were chosen for further study. 2B4.11 responds to the COOH-terminal portion of the antigen pigeon cytochrome *c* (pigeon fragment 81–104) in association with the E^k_β:E^k_α Ia molecule (17), and C10.9 recognizes HEL in the presence of the A^k_β:A^k_α Ia molecule (18). Antigen dose-response curves were performed in the presence of a constant number of B10.A splenic APC, which bear both of these Ia molecules (Fig. 1). Both of the T cell hybrids exhibited a decrease in [^{3}H]thymidine incorporation and a corresponding increase in the amount of IL-2 produced as the concentration of antigen was increased. In fact, similar concentrations of antigen were required to achieve 50% inhibition of [^{3}H]thymidine incorporation and 50% stimulation of IL-2 release. This inversely proportional relationship suggested that the inhibition of [^{3}H]thymidine incorporation was a consequence of cellular activation, and demonstrated that, for these two T cell hybridomas, the sensitivity of this assay in detecting antigenic stimulation was comparable to one that measures the secretion of IL-2.

Inhibition of [^{3}H]Thymidine Uptake Is a Consequence of Receptor Occupancy. A variety of approaches were used to examine the antigen-specificity of the inhibition of [^{3}H]thymidine incorporation. First, titration of the "wrong" antigen (either pigeon fragment 81–104 with C10.9, or HEL with 2B4.11) in the presence of B10.A APC failed to cause either IL-2 release or inhibition of [^{3}H]-thymidine incorporation (data not shown). Second, since these antigens must be "corecognized" with a specific Ia molecule, monoclonal antibodies that bind to the two required Ia molecules were tested for their effect on [^{3}H]thymidine incorporation (Table II). The monoclonal antibody 14.4.4 binds to the E^k_β:E^k_α Ia

TABLE I

T Cell Hybridoma Proliferation and IL-2 Production upon Stimulation with Antigen

T cell	Mouse strain (haplotype)*	Reference	Ia molecule‡	Antigen	Concentration	[^{3}H]Thymidine uptake	IL-2 production	
						cpm	*cpm*	*U*
2B4.11	B10.A (H-2^a)	17	E_β^k:E_α^k	Cytochrome c§	0	41,900	1,100¶	0‖
					3 μM	11,100	32,600	78
					30 μM	5,000	35,400	>128
2H10	B10.A (H-2^a)	17	E_β^k:E_α^k	Cytochrome c	0	84,000	1,300	0
					3 μM	37,500	34,600	10
					30 μM	18,700	35,400	17
2C2	B10.A (H-2^a)	17	E_β^k:E_α^k	Cytochrome c	0	89,600	800	0
					3 μM	71,600	44,100	70
					30 μM	31,100	45,900	120
C10.9	B10.A (H-2^a)	18	A_β^k:A_α^k	HEL	0	65,000	200	0
					30 μg/ml	27,900	31,000	24
					300 μg/ml	11,500	35,700	38
A4.A1.4	B10.A (H-2^a)	18	A_β^k:A_α^k	HEL	0	57,300	3,800	ND
					30 μg/ml	41,600	19,600	
					300 μg/ml	14,300	30,300	
A6.A2.7	B10.A (H-2^a)	18	A_β^k:A_α^k	HEL	0	27,500	4,500	ND
					30 μg/ml	14,600	27,900	
					300 μg/ml	2,500	40,500	
3DO.54.8	BALB/c (H-2^d)	19	A_β^d:A_α^d	Ovalbumin	0	59,000	3,000	ND
					100 μg/ml	10,600	11,000	
					1,000 μg/ml	6,100	18,000	
F9.140	BALB/c (H-2^d)	20	A_β^d:A_α^d	GAT	0	26,800	2,500	ND
					100 μg/ml	18,800	6,600	
					1,000 μg/ml	11,900	27,000	

10^4 T cell hybrids were assessed for [^{3}H]thymidine incorporation and IL-2 production after 24 h of incubation, as detailed in Materials and Methods. The cells should not be compared on the basis of absolute [^{3}H]thymidine incorporation because the data are taken from different experiments.

* Mouse strain and haplotype of the normal T cells used as the fusion partners for BW5147.

‡ The source of splenic APC for each of the specified allelic forms of the Ia molecule are E_β^k:E_α^k, B10.A; A_β^d:A_α^d, B10.D2.

§ Pigeon cytochrome c fragment 81–104 was used in these experiments.

‖ [^{3}H]Thymidine incorporation by CTLL cells in the presence of a 1:4 (25%) dilution of supernatant.

¶ In some experiments, the T cell hybrid supernatants were titered in the presence of CTLL cells to obtain the number of IL-2 units produced, as described in Materials and Methods.

molecule (corecognized by 2B4.11), and the monoclonal antibody 10-2.16 binds to the A_β^k:A_α^k Ia molecule (corecognized by C10.9). The addition of 10-2.16 blocked the inhibition of [^{3}H]thymidine incorporation observed when C10.9 was incubated with HEL, but had no effect when added to 2B4.11 plus pigeon fragment 81–104 (Table II). Conversely, the antibody 14.4.4 blocked the inhibitory effect of the pigeon fragment 81–104 on the [^{3}H]thymidine incorporation of 2B4.11 by ~100-fold, but had no effect on the response of C10.9 to HEL.

Further evidence that it was occupancy of the T cell hybridomas' antigen-specific receptors that resulted in inhibition of [^{3}H]thymidine incorporation was obtained by using the monoclonal antibody A2B4-2, a mouse IgG2a that recognizes an epitope on the antigen receptor of 2B4.11, but that is not on the antigen receptor of C10.9 (see reference 15 and Fig. 4, below). This antibody is an

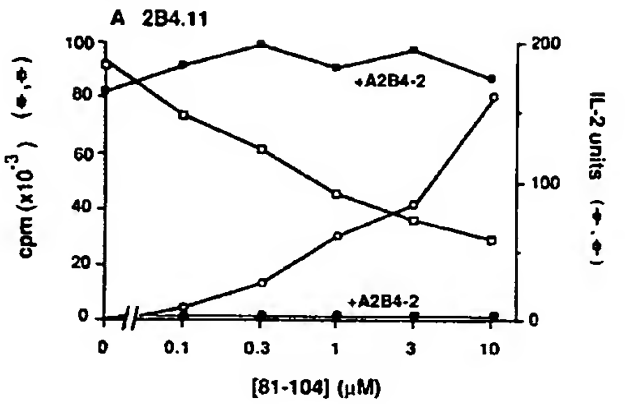

FIGURE 1. Incorporation of [^{3}H]thymidine and secretion of IL-2 as a function of antigenic stimulation. (*A*) 10^4 2B4.11 T cells or (*B*) 10^4 C10.9 T cells were incubated in the presence of 5×10^5 irradiated B10.A splenocytes and varying concentrations of the appropriate antigen. After 24 h, an aliquot of supernatant was removed from each well for the quantitation of IL-2 content (*circles*), [^{3}H]thymidine was added to each well, and [^{3}H]thymidine incorporation by the T hybridoma cells (*squares*) was determined as described in Materials and Methods. In parallel culture wells the antibody A2B4-2 was added at a final concentration of 0.63 μg/ml (*closed symbols*).

extremely potent inhibitor of antigen-stimulated IL-2 release by 2B4.11 (15). A2B4-2 was added to an assay in which 2B4.11 was stimulated with antigen (Fig. 1). The anticlonotypic antibody blocked both the release of IL-2 and the inhibition of [^{3}H]thymidine incorporation. No effect was observed when similar concentrations of A2B4-2 were added to an assay performed with C10.9.

Although the soluble form of the antibody A2B4-2 exhibits no stimulatory effect upon 2B4.11, when it is crosslinked to Sepharose beads it will cause 2B4.11, but not other T cell hybridomas that bear unrelated antigen receptors, to release IL-2 in a dose-dependent fashion (H. Narimatsu and R. Schwartz, personal communication). Table III contains the results of a typical experiment in which A2B4-2 crosslinked to Sepharose was used to stimulate 2B4.11. Whereas soluble A2B4-2 had no stimulatory effect, the addition of A2B4-2 crosslinked to Sepharose beads resulted in both IL-2 release and inhibition of [^{3}H]thymidine incorporation. The antigen-specificity of these responses was demonstrated by

TABLE II

Reversal of Antigen Inhibition with Anti-Ia Monoclonal Antibodies

T cell	Antigen		[^{3}H]Thymidine incorporation after culture with:		
	81-104	HEL	No antibody	10-2.16	14.4.4
	μM	*μg/ml*		*cpm*	
C10.9	0	0	47,700	43,200	46,200
	0	3	30,600	42,800	20,400
	0	30	12,700	58,600	13,400
	0	300	7,700	50,400	7,700
2B4.11	0	0	55,100	52,700	68,300
	0.3	0	37,500	40,000	49,700
	3	0	24,400	32,200	66,500
	30	0	15,600	13,800	32,500

10^4 T cell hybridomas were cultured in triplicate with 5×10^5 irradiated B10.A splenocytes as APC and varying concentrations of the indicated antigen, in the presence or absence of a 1:500 dilution of ascites containing 10-2.16 (anti-A^k_β:A^k_α) or 14.4.4 (anti-E^k_β:E^k_α). Incorporation of [^{3}H]thymidine was determined after 24 h in culture as described in Materials and Methods.

TABLE III

Effect of A2B4-2-coupled Sepharose Beads on 2B4.11 and C10.9

A2B4-2-coupled beads	Soluble A2B4-2	[^{3}H]Thymidine incorporation with T cell hybridoma:		IL-2 production by T cell hybridoma	
		2B4.11	C10.9	2B4.11	C10.9
μl		*cpm*		*U*	
—	−	119,900	145,700	0	0
—	+	113,300	158,500	0	0
0.125	−	66,900	154,000	24	0
0.25	−	64,600	148,000	36	0
0.5	−	58,300	145,200	40	0
1.0	−	44,400	143,000	46	0
2.0	−	37,900	148,700	38	0
2.0	+	112,200	154,300	0	0

10^4 2B4.11 or C10.9 T cells were cultured in duplicate wells in the presence of either soluble A2B4-2 antibody (0.63 μg/ml of purified antibody), varying amounts of A2B4-2-coupled Sepharose beads, or both, in a total volume of 200 μl. After 24 h, 50 μl of supernatant was removed from each well for the determination of IL-2 units, and the cells were pulsed with 1 μCi of [^{3}H]thymidine for 2 h, after which time the wells were harvested and the [^{3}H]thymidine incorporation determined by scintillation counting.

the fact that both were reversed by the addition of soluble A2B4-2. Stimulation of the T cell 2B4.11 with pigeon fragment 81–104 in association with the E^k_β:E^k_α Ia molecule incorporated into lipid planar membranes also resulted in both IL-2 production and growth inhibition (J. D. Ashwell and Barbara Fox, unpublished results). Taken together, these data indicate that the occupancy (or crosslinking) of the antigen-specific T cell receptor in the absence of any signals provided by

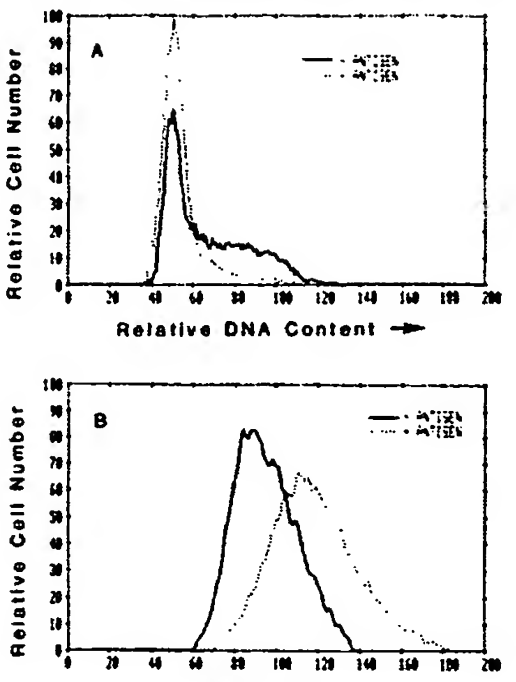

FIGURE 2. Flow cytometric analysis of the effect of antigen stimulation upon T cell hybridoma DNA content and volume. C10.9 T cells were incubated at a concentration of 3×10^5 cells/ml with 5×10^6 irradiated B10.A splenocytes per milliliter in the absence (*solid lines*) or presence (*dotted lines*) of 300 μg/ml of HEL. After 24 h the T hybridoma cells were separated from the APC by density centrifugation and fixed with ethanol. (*A*) DNA content was assessed by staining with propridium iodide. The first large peak represents cells in G_1, those cells with twice the amount of DNA are in G_2 or M, and those cells that are synthesizing DNA are found between the two peaks, in S phase. The fraction of cells in each phase of the cell growth cycle in the absence of antigen was, G_1, 36%; S, 62%; and G_2 plus M, 2%. In the presence of HEL, it was G_1, 78%; S, 20%; and G_2 plus M, 2%. (*B*) Cell size was determined by axial extinction measurements and expressed as arbitrarily defined relative units.

other cells can lead to the inhibition of [^{3}H]thymidine incorporation by T cell hybridomas.

Antigen Stimulation Causes a G_1/S Cell Growth Cycle Block. The incorporation of [^{3}H]thymidine reflects both the quantity and the rate of DNA synthesis, and its decrease could be due to a block anywhere in a cell's growth cycle. To determine the point(s) at which antigen stimulation blocked the cell cycle, C10.9 T cells were incubated with irradiated APC in the presence or absence of HEL. After 24 h the T cell hybridomas were separated from the APC by density centrifugation and analyzed by flow cytometry (Fig. 2). In the absence of specific antigen (Fig. 2*A*, solid line), the DNA profile for C10.9 T cells was typical of rapidly proliferating cells, with ~36% of the cells in G_1, 62% in S, and 2% in G_2 and M phases of the cell cycle. The addition of the antigen HEL to the 24-h culture caused a marked increase in mean cell size (Fig. 2*B*), accompanied by a change in the DNA profile (Fig. 2*A*, broken line), with the majority of the cells (78%) in G_1, 20% in S, and 2% of the cells in G_2 or M phase. The major effect after stimulation with antigen thus seemed to be a cell cycle block at the G_1/S border. Similar data was obtained with the T cell 2B4.11 (data not shown; see

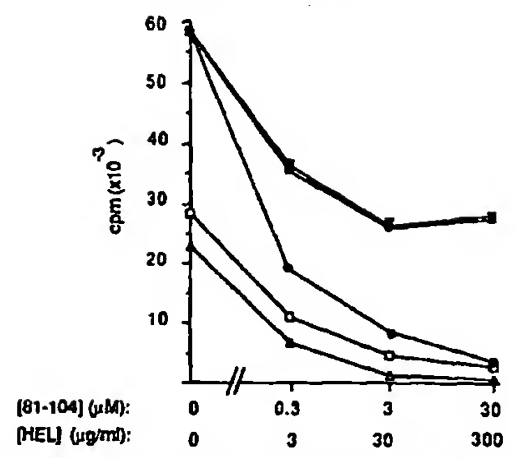

FIGURE 3. The inhibition of [^{3}H]thymidine incorporation is evident only in the stimulated T hybridoma cell. 5×10^3 2B4.11 (□) or C10.9 (△) T cells were assayed for their ability to incorporate the [^{3}H]thymidine after 24 h of culture in the presence of irradiated B10.A splenocytes and varying amounts of antigen. In parallel cultures, 5×10^3 of each of these cells were mixed together and stimulated with either pigeon fragment 81–104 (■), HEL (▼), or both antigens simultaneously (●).

also Fig. 4, below). A second effect of antigen stimulation was to cause some cells in S phase to either slow or cease their synthesis of DNA (Fig. 2*A*). This interpretation is supported by experiments in which cell cycle analysis of antigen-stimulated cells was performed after 24 h and also after 48 h, in the presence or absence of the mitotic inhibitor colcemid. It was observed that those cells found to be in S phase at 24 h had failed to accumulate in M phase by 48 h, suggesting that they were no longer cycling (data not shown).

Inhibition of Growth Is the Result of a Direct Effect upon the Stimulated Cell. The cell cycle block induced by stimulation with antigen could be a direct result of antigen receptor occupancy, or it could be secondary to the binding of soluble substances (such as lymphotoxin) produced by the activated T cell hybrids. To test these possibilities, equal numbers of C10.9 and 2B4.11 were assayed, both independently and when admixed, for the effect of antigen stimulation upon [^{3}H]thymidine incorporation (Fig. 3). When incubated separately, the growth of both T cell hybridomas was inhibited in an antigen dose–dependent fashion. When mixed together in the absence of antigen the amount of [^{3}H]thymidine incorporated was approximately equal to the sum of the individual populations. The addition of either antigen alone resulted in the loss of the contribution made by the appropriate antigen-specific T cell. At maximal stimulation the combined response was reduced by ~50%. Thus, at concentrations of pigeon fragment 81–104 that were shown to cause maximal growth inhibition of 2B4.11 (e.g., 30 μM), the mixed population incorporated ~26,000 cpm, and at the comparable concentrations of HEL (300 μg/ml) the mixed population incorporated 27,000 cpm. In the presence of both antigens, however, [^{3}H]thymidine incorporation by the mixed population was almost completely inhibited. These results suggested that the inhibitory effect of antigenic stimulation was direct, being manifested only in T cells hybridomas in which the antigen receptors were engaged.

To further analyze the potential for a secreted inhibitor of growth, we made

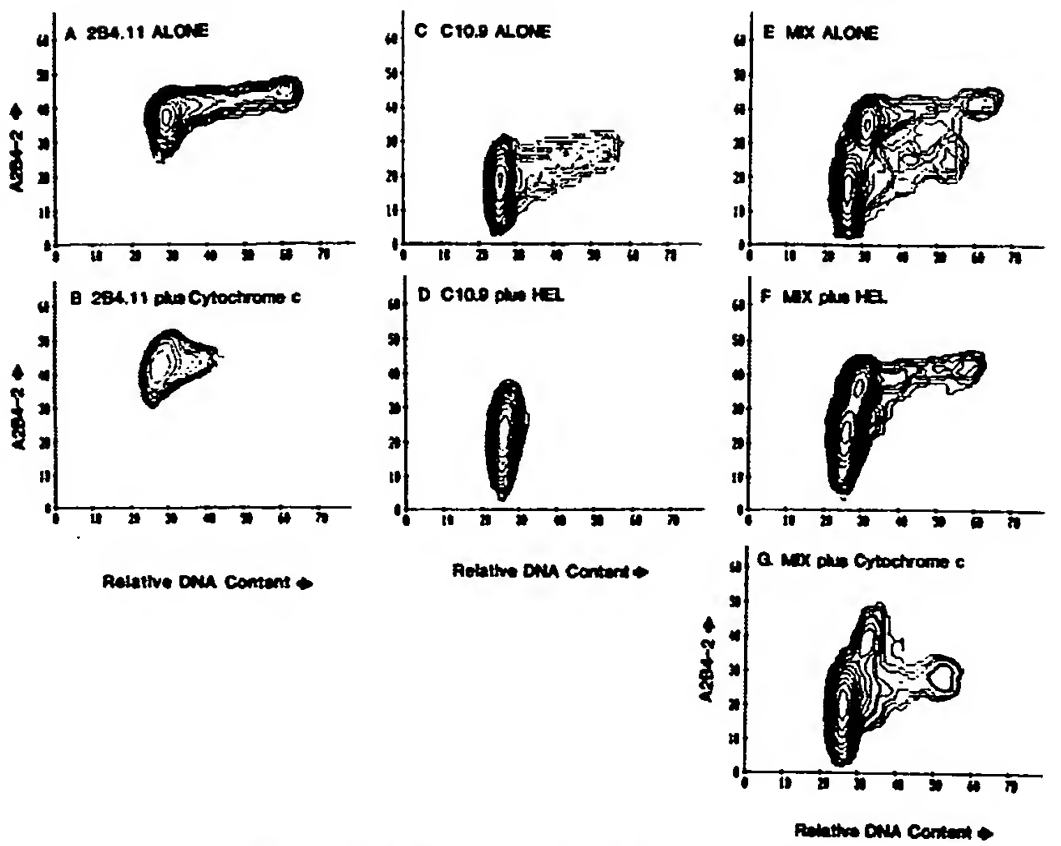

FIGURE 4. Cell growth cycle blockade after antigenic stimulation is evident only in the stimulated T hybridoma cell. (*A* and *B*) 3×10^5 2B4.11 T cells/ml were incubated with 5×10^6 irradiated B10.A splenocytes in the absence (*A*) or presence (*B*) of 100 μM pigeon fragment 81–104. (*C* and *D*) 3×10^5 C10.9 T cells/ml were incubated with 5×10^6 irradiated B10.A splenocytes in the absence (*C*) or presence (*D*) of 300 μg/ml of HEL. (*E–G*) 1.5×10^5 2B4.11 and 1.5×10^5 C10.9 T cells were incubated together (*MIX*) in the absence of antigen (*E*), in the presence of 300 μg/ml of HEL (*F*), or in the presence of 100 μM pigeon fragment 81–104 (*G*). After 24 h the cells were stained with A2B4-2 and FITC–goat anti–mouse Ig so that the two cell populations could be distinguished in mixed cultures.

use of the fact that the monoclonal antibody A2B4-2 can distinguish between 2B4.11 and C10.9. Correlated DNA/immunofluorescence analysis was performed upon a mixture of these two T cells in the presence of no antigen, pigeon fragment 81–104, or HEL (Fig. 4). Each T cell divided rapidly when incubated alone or in the presence of the other T cell (Fig. 4, *A*, *C*, and *E*). Surface staining with A2B4-2 allowed the clear differentiation of the two cell populations, even when they were mixed together (Fig. 4*E*). The addition of pigeon fragment 81–104 to 2B4.11 and HEL to C10.9, resulted in predominantly a G_1/S interface block (Fig. 4, *A* vs. *B*; *C* vs. *D*). The addition of these antigens to the inappropriate cell had no effect on the cell cycle (data not shown). Strikingly, in the mixed population, the addition of HEL blocked the entry into S phase of virtually all C10.9 T cells (A2B4-2^-), while having almost no effect upon the cell cycle of 2B4.11 T cells (A2B4-2^+) (Fig. 4*F*). The reciprocal experiment, in which pigeon fragment 81–104 was used to stimulate the mixed population, demonstrated a marked cell cycle block of the 2B4.11 T cells, with virtually no effect upon the

C10.9 T cells (Fig. 4*G*). This result demonstrates that the major effect of stimulation with antigen is upon the antigen-specific T cell, and suggests that soluble factors alone were not responsible for the cell cycle block.

Effect of Antigen Stimulation on T Cell Growth. To quantitate the effect of antigenic stimulation upon the growth of T cell hybridomas, C10.9 or 2B4.11 T cells were cultured in the presence of either B10.A APC, which bear both Ia molecules required for the presentation of soluble antigen to these two T cell hybrids, or B10.D2 APC, which do not bear the necessary allelic forms of the Ia molecule. The T cell hybridomas were cultured in the presence or absence of the appropriate antigens, and cell counts were performed at 24-h intervals (Fig. 5). C10.9 T cells grown in the presence of B10.A APC without antigen, or in the presence of B10.D2 APC plus HEL, grew with a doubling time of ~12 h (Fig. 5*A*). In contrast, the growth of C10.9 T cells cultured in the presence of B10.A APC and 300 μg/ml of HEL was markedly inhibited early in the course of the culture. After a lag of ~4–5 d, the number of viable cells began to increase exponentially, with a doubling time virtually identical to that displayed by the unstimulated C10.9 T cells. A similar result was obtained when this experiment was performed with 2B4.11 T cells (Fig. 5*B*). As with C10.9, the unstimulated 2B4.11 cells grew rapidly, with a doubling time of ~13.5 h. The growth of 2B4.11 T cells cultured in the presence of B10.A APC and 30 μM pigeon fragment 81–104 was markedly slowed early in the course of the culture. Again, after a lag of ~4 d, the number of viable cells in the wells that had been appropriately stimulated began to increase exponentially, with a doubling time similar to that of the unstimulated 2B4.11 population. In another experiment (Fig. 5*C*), the effect of different concentrations of antigen on cell growth was determined. Little growth of 2B4.11 was evident in the presence of 3 μM pigeon fragment 81–104 until day 3, whereas its growth was delayed ~1 d longer in the presence of 100 μM antigen (the growth curve for 30 μM antigen [not shown] was superimposable with that for 100 μM antigen).

Functional assays were performed to determine the responsiveness of those T cells that had expanded after a lag period, despite the presence of antigen. From the experiment displayed in Fig. 5*A*, 10 independent wells from which C10.9 cells had arisen after culture in the presence of B10.A APC and 300 μg/ml of HEL were selected at random and expanded. All 10 of these sublines were substantially less sensitive to antigenic stimulation than was the parent C10.9 clone, and on average appeared to be at least 100-fold more difficult to activate, as judged both by IL-2 production and growth inhibition (Table IV). The decreased sensitivity to antigen was a stable property. When these 10 C10.9 sublines were retested after 14 d (~28 generations) with HEL concentrations of up to 300 μg/ml, their responses were very similar to those given in Table IV, with a mean decrease in [^{3}H]thymidine incorporation of only 57% at the highest concentration of antigen (data not shown). Thus, it appeared that the original C10.9 line contained a subpopulation(s) that was a relatively poor responder to HEL, and whose growth was therefore not completely inhibited in the presence of antigen. Given a doubling time of 12 h (Fig. 5*A*), it can be determined that an antigen-resistant subpopulation of only 0.5% in the original C10.9 line could account for the growth that was observed in the antigen-stimulated wells after 5

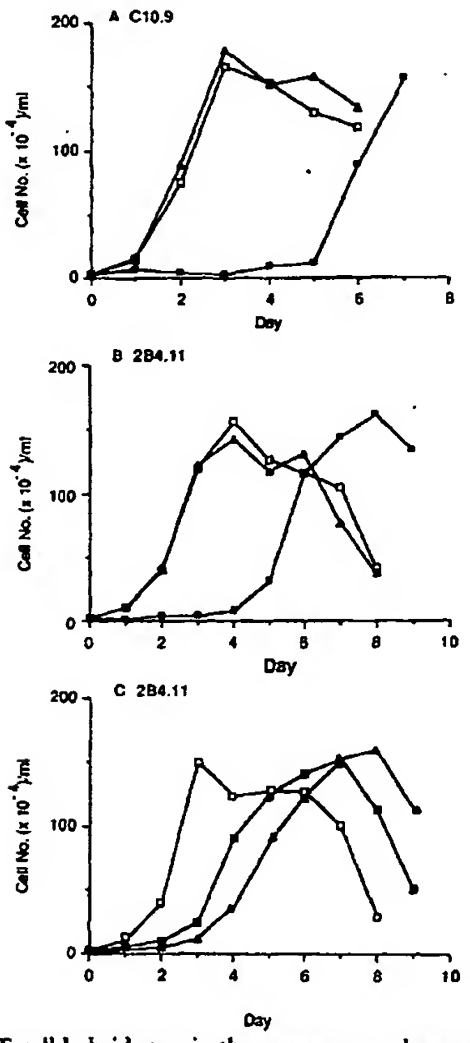

FIGURE 5. Growth of T cell hybridomas in the presence or absence of antigen/MHC. (*A*) 5 × 10^5 C10.9 T cells were incubated with 5 × 10^5 irradiated B10.A or B10.D2 splenocytes in the presence or absence of 300 μg/ml HEL. (□), B10.A APC, no antigen; (■), B10.A APC, HEL; (△), B10.D2 APC, HEL. (*B*) 5 × 10^5 2B4.11 T cells were incubated with 5 × 10^5 irradiated B10.A or B10.D2 splenocytes in the presence or absence of 30 μM pigeon fragment 81–104. (□), B10.A APC; no antigen; (■), B10.A APC; 81–104; (△), B10.D2 APC; 81–104. (*C*) 5 × 10^5 2B4.11 T cells were incubated with 5 × 10^5 irradiated B10.A splenocytes and either no, 3 μM, or 100 μM pigeon fragment 81–104. (□), No antigen; (■), 3 μM 81–104; (▲), 100 μM 81–104. Replicate wells were resuspended daily and viable cell recovery assessed by light microscopic examination in the presence of trypan blue dye. Each point represents the arithmetic average of triplicate determinations.

d. Since both measurements of T cell activation (IL-2 production and growth inhibition) were diminished, these sublines might have consisted of clones that had lost a substantial portion of their antigen-specific receptors. It was not possible to test this directly, because no anticlonotypic antibody exists for this T cell. It was also possible that other molecules involved in the response to antigen might have been lost by the resistant sublines. Indeed, flow cytometric analysis performed with the anti-L3T4 antibody, GK1.5, revealed that, whereas the large majority (91%) of the parent C10.9 T cells stained intensely with this antibody,

TABLE IV

Responsiveness of C10.9 Sublines to Antigen/MHC

Number of C10.9 sublines tested	Maximal [^{3}H]thymidine incorporation (%) with HEL (μg/ml):					
	0	1	3	10	30	100
A.						
1 (no selection)*	100	50	34	41	21	8
10	100	95 ± 5‡	100 ± 5	97 ± 5	91 ± 6	72 ± 5
B.	IL-2-production (U)					
1 (no selection)*	0	3.5	3.8	6	10	15
10	0	0.05 ± 0.05‡	0.4 ± 0.27	1.1 ± 0.4	1.9 ± 0.37	3 ± 0.56

Ten of the antigen-containing microtiter wells from the cell growth experiment shown in Fig. 5*A*, in which C10.9 was cultured in the presence of HEL (300 μg/ml), were collected on day 6, and the cells were expanded in growth medium alone. 4 d later, each C10.9 subline was tested for its ability to produce IL-2 and to incorporate [^{3}H]thymidine upon stimulation with HEL in the presence of irradiated B10.A APC.

* The original C10.9 clone, carried in vitro, from which the sublines were derived.

‡ Mean ± SEM.

TABLE V

Responsiveness of 2B4.11 Sublines to Antigen/MHC

Number of 2B4.11 sublines tested	Selecting antigen concentration	Maximal [^{3}H]thymidine incorporation (%) with pigeon fragment 81–104 (μM):					
		0	0.3	1	3	10	30
A.							
1*	None	100	34	22	10	7	5
5‡	None	100	34 ± 3§	24 ± 2	12 ± 2	12 ± 2	5 ± 2
5	3 μM	100	43 ± 3	30 ± 4	24 ± 2	15 ± 2	13 ± 3
5	100 μM	100	40 ± 3	32 ± 2	27 ± 4	19 ± 2	18 ± 1
B.		IL-2 production (U)					
1*	None	0	20	32	40	40	35
5‡	None	0	16 ± 8§	33 ± 10	57 ± 9	71 ± 11	69 ± 7
5	3 μM	0	17 ± 1	33 ± 4	36 ± 6	43 ± 4	43 ± 5
5	100 μM	0	10 ± 2	13 ± 2	17 ± 2	13 ± 2	15 ± 3

2B4.11 sublines were established from independent microtiter wells on day 4 of the cell growth experiment shown in Fig. 5*C*, in which 2B4.11 was cultured in the absence or presence of pigeon fragment 81-104 (3 or 100 μM). 7 d later each 2B4.11 subline was tested for its ability to produce IL-2 and to incorporate [^{3}H]thymidine upon stimulation with pigeon fragment 81-104 in the presence of irradiated B10.A APC.

* The original 2B4.11 clone, carried in vitro, from which the sublines were derived.

‡ Sublines derived from microtiter wells in which no antigen was added.

§ Mean ± SEM.

only 9% of the C10.9 cells that had grown in antigen-containing wells bore the L3T4 molecule.

An analysis performed with 2B4.11 gave a similar, but quantitatively different, result (Table V). Sublines of 2B4.11 were established from the experiment displayed in Fig. 5*C*. Five sublines that arose in the absence of antigen were as sensitive to antigenic stimulation, as was the original 2B4.11 clone carried in

vitro, both in terms of their production of IL-2 and the inhibition of growth. At their maximal response, sublines of 2B4.11 that had been grown in the presence of either 3 μM or 100 μM pigeon fragment 81–104 produced considerably less IL-2 than did the sublines that had not been selected with antigen. The ability of antigen to inhibit their incorporation of [^{3}H]thymidine was also somewhat decreased, especially at high concentrations of antigen (i.e., the plateau inhibitory response was less). Flow cytometric analysis and cell surface staining with the anticlonotypic antibody A2B4-2 showed that stimulation with increasing doses of antigen did indeed select for receptor-negative variants of 2B4.11. On average, the fraction of receptor-negative 2B4.11 T cells, as a function of the concentration of pigeon fragment 81–104 used to select them, was: 0 μM, 3%; 3 μM, 6%; 100 μM, 18%. The expression of L3T4 was not a useful parameter to follow for these cells, since the parent clone bears negligible amounts of this molecule (data not shown).

Failure to Reverse Cell Cycle Block 24 h after Removal of Antigen. These data suggest that selection of relatively antigen-resistant cells does occur in the presence of antigen, but also argue that in some cases a substantial number of antigen-responsive cells can survive. This result is compatible with the hypothesis that the cell cycle block is reversible, presumably allowing the inhibited cells to resume their growth as the APC in the cultures die and become nonfunctional. An alternative possibility is that, because of the requirement that a T cell must encounter an antigen-presenting cell for stimulation to occur, a small number of antigen-responsive 2B4.11 T cells may simply have escaped sufficient stimulation to be inhibited (with a doubling time of 13.5 h the survival of ~1.5% and 6% of the antigen-stimulated 2B4.11 T cells could account for the growth patterns depicted in Fig. 5*B* and *C*, respectively).

To directly test whether the cell cycle block was reversible, flow cytometric cell cycle analysis was again used (Fig. 6). The T cell C10.9 was incubated with irradiated B10.A APC in the presence or absence of HEL. After 24 h the T cells from both groups were separated from the APC by density centrifugation, washed thoroughly, and placed back in culture in the presence of the anti-Ia antibody 10-2.16 to ensure that any residual antigen-pulsed APC would not be stimulatory (see Table II). In a separate antigen-stimulated group, the medium was changed at 24 h and fresh antigen was added to maintain the stimulation. After another 24 h, which would be enough time for two doublings of untreated C10.9 T cells, cell growth cycle analysis was performed on each group (Fig. 6). The profile of cells incubated for 48 h in the absence of antigen was found to contain ~58% of the cells in G_1, 40% in S, and 2% in G_2 plus M (Fig. 6*A*). Incubation in the presence of continuous antigen inhibited the movement of cells from G_1 to S phase (Fig. 6*B*). ~71% of the cells were found to be in G_1, 27% in S, and 2% in G_2 plus M. A significant number of cells remained in S phase, indicating either an incomplete G_1/S block or an S phase cell cycle block, as suggested above. The removal of the APC and antigen at 24 h had little if any effect upon the degree of the G_1/S cell cycle block measured at 48 h, because this group was found to have a cell cycle profile nearly identical to that of cells that were maintained in continuous antigen for 48 h (Fig. 6, *B* vs. *C*), with 86% in G_1, 13% in S, and 1% in G_2 plus M. It can also be seen that the characteristic

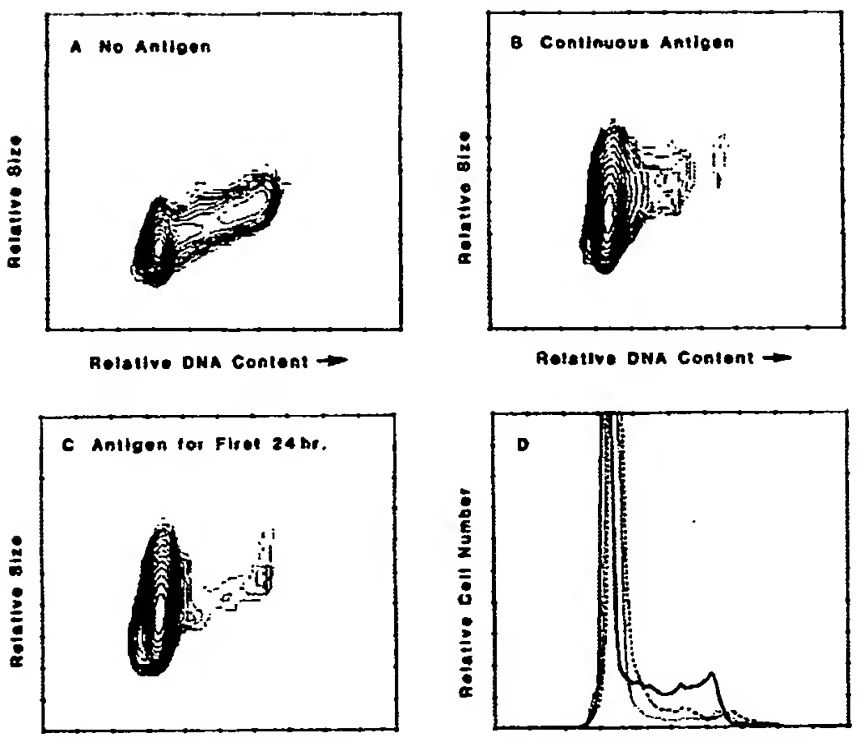

FIGURE 6. Lack of reversibility of the G_1/S block after 24 h. 10^5 C10.9 T cells/ml were incubated with 5×10^6 irradiated B10.A splenocytes in the presence (*B* and *C*) or absence (*A*) of 300 μg/ml HEL. (*A*) After 48 h the T cells were separated from the irradiated splenocytes by density centrifugation, fixed with ethanol, and analyzed for DNA content. (*B*) 24 h after the initiation of the cultures the medium was removed and replaced with fresh complete medium containing 300 μg/ml HEL. After another 24 h the T cells were isolated by density centrifugation and cell cycle analysis performed. (*C*) 24 h after the initiation of the cultures the T cells were isolated by density centrifugation, washed three times, and placed back in culture in fresh complete medium in the absence of antigen. A 1:500 final concentration of 10-2.16-containing ascites was added to each tube to ensure that if a small number of APC were carried over into the second cuture they would be unable to stimulate the T cells (see Table II). After an additional 24 h of incubation, cell cycle analysis was performed. (*D*) Superimposed profiles of the relative DNA content of the three C10.9 T cell populations: no antigen (*solid line*), continuous antigen (*dashed line*), and antigen removed at 24 h (*dotted line*).

cell size increase persisted in these cells 24 h after the removal of antigen. Interestingly, the population of cells from which antigen was removed at 24 h had fewer cells in S and correspondingly more in G_1, suggesting that the S block was at least partially reversible, its release allowing some of these cells to resume cycling and to eventually be blocked at the G_1/S interface. Thus, for at least the large majority of the T cells, after 24 h of exposure to antigen the G_1/S cell cycle block appeared to be irreversible up to 24 h later. Similar results were obtained with the T cell hybridoma 2B4.11 (data not shown). Taken together, these results are compatible with the hypothesis that the eventual growth of T cell hybridomas after stimulation with antigen represents two phenomena: (*a*) the selection of T cell variants that are less sensitive to antigenic stimulation than the parent population (most evident for C10.9, Table IV), and (*b*) the growth of a small

population of typically responsive T cells that were insufficiently stimulated in the initial culture to develop a cell cycle block (e.g., 2B4.11, Table V).

Discussion

T cell hybridomas have been used extensively in the study of the requirements for, and the consequences of, T cell activation. One of the first, and still the most common, assays used to assess the response of a T cell hybridoma to a given stimulus is to measure the culture supernatants for the presence of IL-2 (23). Whereas most T cell hybrids do not produce IL-2 spontaneously, they secrete it upon stimulation with a variety of agents, including lectin, antigen/MHC, and crosslinked anticlonotypic antibodies (6, 23–25). In addition to IL-2, activated T cell hybrids can produce a large variety of soluble factors upon stimulation, including BSF-1 and other B cell proliferation and differentiation factors, IFN-γ, colony-stimulating factors, and lymphotoxin (26–30). In the present report we have demonstrated that T cell hybridomas activated via the antigen-specific receptor also develop a block in their cell growth cycle. The signals for growth inhibition appeared to share the same recognition requirements as the signals for lymphokine release. Further, the ability of anticlonotypic antibodies crosslinked to an insoluble matrix, or antigen plus Ia molecules incorporated into an artificial lipid planar membrane, to cause both IL-2 production and growth inhibition demonstrated that no cell other than the responding T cell hybrid was required to manifest this phenomenon.

The present report adds the antigen-specific T cell receptor to the growing list of receptors whose occupancy, while functioning to promote growth in normal cells, inhibits the growth of some transformed cell lines. For example, IL-2 is a well-described stimulus of proliferation in normal T cells (31). It was recently observed that some spontaneously dividing human T cell leukemia virus–transformed T cells ceased to grow in the presence of recombinant IL-2 (10). In addition, exposure of a murine T cell line, transfected with the human IL-2 receptor gene, to recombinant IL-2 resulted in inhibition of its growth (11). Another example is epidermal growth factor (EGF), which usually functions to stimulate anchorage-independent growth in a variety of nontransformed cells (32). Normally mitogenic concentrations of EGF inhibit the growth of the rat pituitary cell line GH_4C_1 (33), and the human epidermoid carcinoma A431, a cell that bears an unusually high level of EGF receptors (8). A closely related hormone, transforming growth factor type β (TGF-β), has also been found to inhibit the anchorage-independent growth of a variety of tumor cells (9, 34) as well as normal IL-2-stimulated human T cells (35).

There is also a striking parallel between the effect of antigen stimulation on normal T cells and their hybrid counterparts and the effect of another lymphocyte-specific stimulus, antiimmunoglobulin (anti-Ig), on normal B cells and some B cell tumors. In normal resting murine B lymphocytes, crosslinking of surface Ig with an appropriate anti–mouse Ig causes an increase in intracellular Ca^{2+}, the breakdown of phosphatidylinositols, and the movement of resting cells into the cell growth cycle (36–38). Anti-Ig treatment of some spontaneously dividing B cell tumors, such as WEHI 231 (39, 40) and BCL_1 (41), on the other hand, results in growth inhibition. In the case of WEHI 231 this is associated with a

G_1/S cell cycle block that, after ~24–48 h, becomes irreversible and is followed by cell death (42). Since WEHI 231 bears the phenotype of an immature B cell, this phenomenon has been suggested as a model for the induction of tolerance in immature B lymphocytes (42, 43). Bearing in mind that the T cell hybridomas used in the present report were prepared by fusing antigen-primed peripheral T cells to a thymoma cell line, it is possible that the antigen-dependent cell cycle block of T cell hybridomas may be related to tolerance induction in immature T lymphocytes. This speculation obviously requires a considerably more thorough understanding of how cellular activation via the antigen receptor occurs before it can be directly examined.

In this regard it is worth considering the nature of the T cell growth cycle block induced by antigenic stimulation. The major block was found to be at the G_1/S interface, with a second block in S phase itself. Removing the stimulus after 24 h and waiting another 24 h before analysis allowed some of the cells that were trapped in S to resume cycling and to be subsequently blocked at G_1/S. The data presented in this study cannot exclude the possibility that the G_1/S block was reversible, but its release required more than 24 h in the absence of antigen. It is also formally possible that the eventual growth of some antigen-responsive 2B4.11 T cells (Table V) was due to a leaky G_1/S block, although this would seem unlikely in view of the fact that the T cells that eventually grew in the presence of continuous antigen did so with a doubling time identical to that of unstimulated cells (Fig. 5). The simplest interpretation of the data is that the cell cycle block is irreversible, and that the T cells that were found to grow after a lag period represented either a small fraction that had escaped stimulation, or those cells that, for any of a variety of reasons, were difficult to stimulate with antigen/MHC. It is interesting to note that exposure of a variety of murine T cell neoplasms, including BW5147 (44, 45) and a murine T cell hybridoma (23), to lectins such as concanavalin A and phytohemagglutinin, has been shown to lead to a decrease in DNA and RNA synthesis and eventually cell death. The growth of both 2B4.11 and C10.9 was also profoundly diminished after 24 h of coculture with concentrations of concanavalin A that induced maximal IL-2 production from these cells (unpublished observation). It is possible that lectin-mediated cellular activation, perhaps by signalling through the antigen receptor/T3 complex (46), delivers the same growth inhibitory signals as does antigen/MHC, and may provide a means of studying this phenomenon in T cell neoplasms for which the appropriate antigen/MHC combination is unknown.

A question of particular interest is how does antigen receptor occupancy result in cell cycle blockade of T cell hybridomas? Experiments in which two T cell hybridomas of different antigen specificities were cultured together in the presence of a single antigen demonstrated that the cell cycle block occurred in only the T cell specific for that antigen (Figs. 3 and 4). It would therefore seem unlikely that the T cell hybridomas produced, and were in turn inhibited by, toxic lymphokines. However, these experiments do not rule out the possibility that antigen activation resulted in two distinct events: the secretion of an inhibitory lymphokine, and the expression of the receptor for that lymphokine. One candidate for such a lymphokine/receptor pair would be the IL-2/IL-2 receptor combination (10, 11). We have ruled out this particular possibility by

the demonstration that the T cell hybridomas used in this study have few if any IL-2 receptors, even when activated with antigen/MHC, and that the addition of anti-IL-2 receptor antibodies to the cultures failed to prevent the growth inhibition caused by antigen activation (unpublished results). However, the possibility still exists that stimulation with antigen induced the expression of some other inhibitory lymphokine and its receptor.

Finally, for completeness, it should be mentioned that some hematopoietic neoplasms, such as erythroleukemia cells, can be induced to terminally differentiate and thereby cease their uncontrolled growth, by a wide variety of chemical (e.g., dimethylsulfoxide, butyrate) and physical (e.g., x and UV irradiation) agents (47). Some myeloid leukemic cells can be similarly induced to differentiate in the presence of colony stimulating factors (48). In view of the fact that normal effector T lymphocytes do not seem to undergo terminal differentiation (e.g., mature and functionally competent T cells seem to have a virtually unlimited capacity to renew themselves in vitro upon repetitive antigenic stimulation), and that antigenic stimulation of T cell hybridomas results in an apparently irreversible cell cycle block and death, as judged by the release of ^{51}Cr (data not shown), it seems unlikely that such an event accounts for the inhibition of growth in T cell hybridomas. Furthermore, differentiation of hematopoietic cells is accompanied by a decrease in singular cell size (49); the increase in cell size accompanying the G_1/S block reported in the present study more likely reflects unbalanced growth leading to cell death, such as that due to deoxyadenosine G_1 arrest of leukemic T cells (50).

The antigen-driven growth arrest of antigen-specific T cell hybridomas may have some useful applications. As demonstrated in Fig. 1, as long as the T cell hybrid population is fairly uniform in its ability to respond to antigen, the measurement of the inhibition of growth is as sensitive and quantitative as the measurement of IL-2 release. Moreover, the growth inhibition assay has a number of distinct advantages over lymphokine assays; it is more rapid (requiring ~24 h vs. 48 h), does not require sequential cell cultures, and most importantly, obviates the need to serially dilute supernatants to achieve quantitative results. It should be noted, however, that because IL-2 is not produced by unactivated T cell hybrids (i.e., the background activity is low), the detection of IL-2 in a nonhomogeneous population might be more sensitive than the assessment of growth inhibition. For example, the detection of a low level of IL-2 might convincingly indicate that at least some small portion of the cells in a mixed population had responded, whereas a small decrease in the incorporation of [^{3}H]thymidine might be considered insignificant because of the high background, resulting in a false negative.

Another potential use for the growth inhibition of antigen-stimulated T cell hybrids is the selection of mutants in the activation/growth inhibition pathway. As found with C10.9 and 2B4.11, small subpopulations that are relatively resistant to activation with antigen/MHC can continue to grow in the presence of cells that are prevented from proliferating, although the experience with 2B4.11 suggests that multiple rounds of selection may be necessary to allow substantial expansion of such clones. Therefore, it should be possible to treat a homogeneous in vitro line with mutagens and select for T cell hybridomas whose

growth is no longer inhibited by antigen/MHC. As demonstrated in this report, such mutants and/or phenotypic variants should include T cells that have lost molecules associated with the recognition of antigen (as with the antigen-specific T cell receptor on 2B4.11, and the L3T4 molecule on C10.9). They would also probably include cells in which the loss or modification of other molecule(s) involved in antigen recognition and/or signal transduction (perhaps such as elements of the T3 complex) had occurred, and might allow the separation of the IL-2 secretory response from the growth inhibitory response. The generation of such cells would be useful in the study of how antigen receptor occupancy leads to lymphokine release and growth inhibition. Finally, the finding that the activation of a variety of independently-derived T cell hybridomas can arrest their growth raises the intriguing possibility that a therapeutic role might be found for similar treatment of antigen-specific T cell tumors in vivo. Studies are currently underway in our laboratory to investigate the potential of such treatment in a model murine tumor system.

Summary

Stimulation of antigen-specific T cell hybridomas with the appropriate antigen/MHC combination, at concentrations that resulted in the secretion of the lymphokine interleukin 2, resulted in a dose-dependent decrease in both [^{3}H]-thymidine incorporation and cell growth. Flow cytometric studies demonstrated that stimulation with antigen resulted in a cell cycle block that was most evident at the G_1/S border, and mixing studies revealed that bystander T cells of different antigen specificities were not affected. For at least the large majority of T cells, the G_1/S cell cycle block appeared to be irreversible after 24 h of exposure to antigen. This cell cycle block may be useful as a rapid and quantitative measure of T cell hybridoma activation, as a means of selecting T cell hybridomas that have functional alterations in the reception of stimulatory signals, and may serve as a model of the induction of tolerance in immature T cells.

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CD44 (Pgp-1) Inhibits CD3 and Dexamethasone-Induced Apoptosis

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Anti-CD3 monoclonal antibodies (MoAbs) and glucocorticoid hormones (GCH) induce apoptosis in immature thymocytes and peripheral T lymphocytes. This process is inhibited by a number of growth factors, including interleukin-2 (IL-2), IL-3, and IL-4, indicating that signals generated by membrane receptors can modulate the survival of lymphoid cells. To investigate whether signals activated by adhesion receptors have a similar activity, we analyzed the effect of CD44 (Pgp-1) adhesion molecule receptor stimulation on T-cell apoptosis induced by three stimuli (anti-CD3 MoAbs, dexamethasone [DEX] treatment, and exposure to ultraviolet irradiation [UV]) on a 3DO T-cell line. The results show that CD44 engagement, either by hyaluronic acid (HA) or anti-CD44 MoAbs, inhibits DNA fragmentation and apoptosis induced by DEX and anti-CD3 MoAbs, whereas that induced by UV, a p53-dependent phenomenon, was not inhibited. Furthermore, the antiapoptotic effect exerted through CD44 activation does not seem related to overexpression of bcl-2 or to have appreciable effects on cell proliferation. Our results indicate that adhesion molecules modulate T-cell survival by counteracting apoptosis induced by DEX or anti-CD3 MoAbs.

APOPTOSIS (programmed cell death) is a common mechanism often triggered by different environmental stimuli.[1-4] It is operative in tissue remodeling and involution during embryogenesis, cell growth control in adult life, various central nervous system (CNS) degenerative diseases, and neoplastic cell growth regulation.[5-8] It takes the form of a cascade of specific biochemical and morphologic events. The most relevant is activation of endogenous endonuclease, which is responsible for internucleosomal DNA fragmentation.[9,10]

Apoptosis is also important in T-cell repertoire development.[11-13] These cells are controlled by a complex process that includes both positive and negative selection. Negative selection is due to apoptosis activated through antigen–T-cell receptor (Ag-TCR) interaction.[14] It has been suggested that glucocorticoid hormones (GCH) and cytokines are also critical regulators of T-cell development.[10,15,16] Interleukins (ILs) and GCH may contribute to the effects that follow this interaction and lead to positive (antiapoptotic and/or proliferative) and/or negative (apoptotic) signals. IL-1, IL-2, and IL-4, in fact, protect thymocyte subpopulations and more differentiated T cells from CD3- or GCH-induced apoptosis.[16-20] Further signals may be involved in regulating the Ag-TCR/CD3-driven apoptosis of T lymphocytes, including those activated by adhesion receptors.[21,22]

CD44 (Pgp-1) is a multifunctional cell surface glycoprotein involved in the homing of circulating lymphocytes to lymph nodes and in lymphocyte-epithelial interaction. It modulates lymphocyte adhesion and activation and acts as a major receptor for hyaluronic acid (HA), an extracellular matrix component.[23-29]

CD44 stimulation by crosslinked anti-CD44 monoclonal antibodies (MoAbs) has been shown to activate both mouse and human T-cell systems and modulate IL-2 production in mouse T lymphocytes.[30-34] This study shows that such stimulation by MoAbs or HA protects T cells from CD3- and dexamethasone(DEX)-induced apoptosis. Apoptosis inhibition was observed when cells were treated in the presence of apoptotic stimuli as well as by pretreatment. Protection was dose-dependent, did not correlate with bcl-2 expression, and was not active against the ultraviolet radiation (UV)-induced apoptosis shown to be dependent on p53 expression.[35] These results suggest that adhesion molecules may partly contribute to T-cell development by modulating cell survival.

MATERIALS AND METHODS

Cell suspensions. A $CD3^+$, $CD4^+$, $CD2^+$, $CD44^+$ subline of the OVA-specific hybridoma T-cell line 3DO[36]; obtained in our laboratory, was used for the experiments. As evidenced by reverse transcription-polymerase chain reaction (RT-PCR) analysis, this subline expresses only the smallest standard isoform of CD44 (CD44s).

Thymocytes were obtained from 4- to 5-week-old C3H/HeN mice purchased from Charles River (Corsico, Milan, Italy). The animals were killed by cervical dislocation, and the thymuses were teased in RPMI-1640 medium.

Cells maintained in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10 μmol/L Hepes buffer were centrifuged at preestablished times at 200*g* for 10 minutes, washed, and adjusted to the desired concentrations (see below). Recovery after 20-hour incubation at 37°C with medium (with no further treatment or pretreatment) was between 70% and 85%. Viability (evaluated by trypan blue exclusion) ranged from 85% to 90%.

Antibody crosslinking and cell treatment. Hamster antimouse CD3 (anti-CD3ε, clone 145-2C11; Pharmingen, San Diego, CA) MoAbs at a concentration of 1 μg per well and/or rat antimouse CD44 (clone IM7; Pharmingen) MoAbs, which recognize nonpolymorphic determinants of CD44 glycoprotein,[37] at the concentrations indicated in the figures, suspended in 1 mL phosphate-buffered saline (PBS), were allowed to adhere in flat-bottomed, high-binding, 24-well plates (Costar, Cambridge, MA) at 4°C. After 18 hours, plates coated with MoAbs were washed, incubated at 37°C for 2 hours with PBS supplemented with 10% FCS, and washed again. Hybridoma T cells or thymocytes were then plated at a concentration of 5×10^5 cells per well and incubated at 37°C for 20 hours or as indicated in the figure legends. Some cultures were performed in the presence

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EXHIBIT 2

Fig 1. Optical microscopy of Giemsa-stained 3DO cells after 20 hours of in vitro culture on 24-well plates with medium alone (A, control) or coated with anti-CD3 MoAbs (1 μg/mL; B). (B) Fragmentation of nuclei into apoptotic bodies is evident in some cells (arrows).

of 100 nmol/L DEX (Sigma, St Louis, MO). In some experiments, cells were pretreated by culturing for 1 hour in anti-CD44–coated plates and then treated by adding DEX or replated in anti-CD3 MoAb-coated plates. Rat antimouse IgG2b MoAbs (Pharmingen; clone R35-38) with the same isotype of anti-CD44 MoAbs were used as controls (CONTROL AB in figures). Cells recovered after culture were used to evaluate cell death and proliferation.

HA (Calbiochem, La Jolla, CA) was allowed to adhere by incubating at 37°C on flat-bottomed, high-binding, 24-well plates (Costar) and then at 4°C for 2 hours. Nonadherent compound was removed by extensive washing. In the experiments in which anti-CD44 was used in soluble form to compare with the effect of adherent MoAbs, the antibody was added to the plates after pretreatment with PBS supplemented with 20% FCS.

UV irradiation. In some experiments, 2×10^5 cells cultured in 24-well plates were suspended in medium, supplemented with 10% FCS and 10 μmol/L HEPES, and exposed to different doses of UV rays from a UV Stratalinker (model 1800; Stratagene, La Jolla, CA). After washing, the cells were recultured on CD44 MoAb-coated plates, and apoptosis was evaluated after 20 hours as described below.

DNA labeling technique and flow cytometry. Apoptosis was measured by flow cytometry as described elsewhere.[38] After culturing, cells were centrifuged, and the pellets were gently resuspended in 1.5 mL hypotonic propidium iodide solution (PI; 50 μg/mL in 0.1% sodium citrate plus 0.1% Triton X-100; Sigma). The tubes were kept at 4°C in the dark overnight. The PI-fluorescence of individual nuclei was measured by flow cytometry with standard FACScan equipment (Becton Dickinson, Mountain View, CA). The nuclei traversed the light beam of a 488-nm Argon laser. A 560-nm dichroid mirror (DM 570) and a 600-nm band pass filter (band width, 35 nm) were used to collect the red fluorescence due to PI DNA staining, and the data were recorded in logarithmic scale in a Hewlett Packard (HP 9000, model 310; Palo Alto, CA) computer. The percentage of apoptotic cell nuclei (subdiploid DNA peak in the DNA fluorescence histogram) was calculated with specific FACScan research software (Lysis II; Becton Dickinson).

DNA electrophoresis. DNA electrophoresis was performed using 10 million cells per group. Cells were washed, centrifuged at 200*g* for 10 minutes, and dissolved in hypotonic lysing buffer (100 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA, 1% sodium dodecyl sulfate [SDS], 200 μg/mL proteinase K, pH 7.5). Samples were extracted once with phenol plus chloroform (1:1, vol/vol) and then with chloroform. RNase A (100 μL, at the concentration of 2.5 μg/mL) was added to each sample. DNA was extracted with phenol plus chloroform and then with chloroform and recovered by centrifuging after overnight precipitation at −20°C in two volumes of ethanol in the presence of 0.3 mol/L Na acetate. Pellets were air-

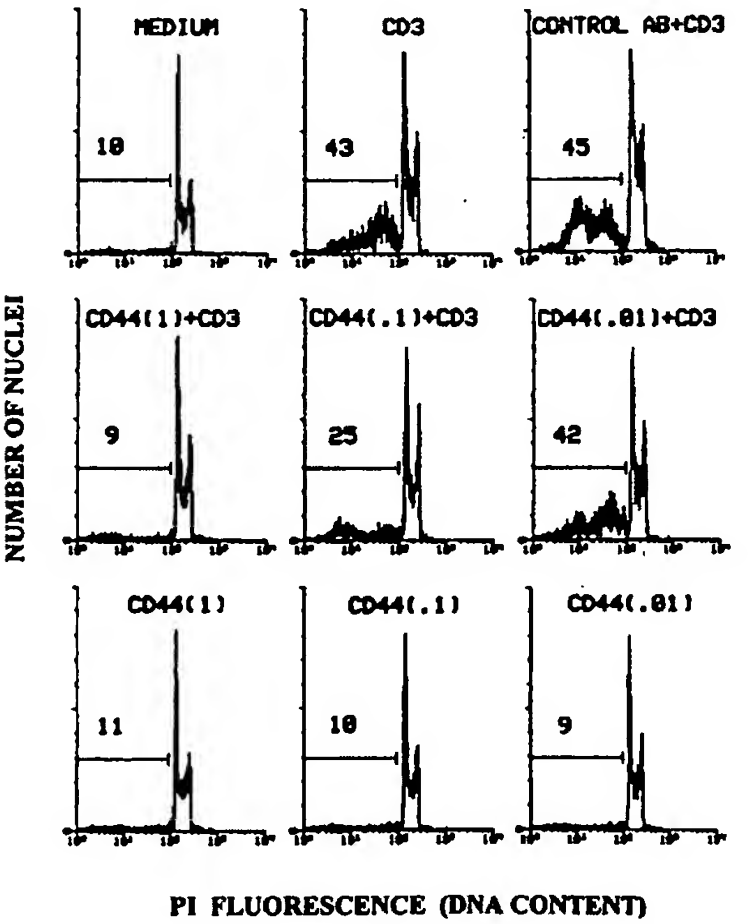

Fig 2. Flow cytometric analysis of PI-stained 3DO nuclei after 20 hours of in vitro culture in 24-well plates with medium alone or coated with anti-CD3 (1 μg/mL) and/or different concentrations (numbers in parentheses) of anti-CD44 MoAbs (MoAbs adhered as described in Materials and Methods). Numbers above histograms indicate the percentage of apoptotic nuclei (broad hypodiploid peak) in a representative experiment. Control AB, MoAbs, with the same isotype of anti-CD44 MoAbs, used as control.

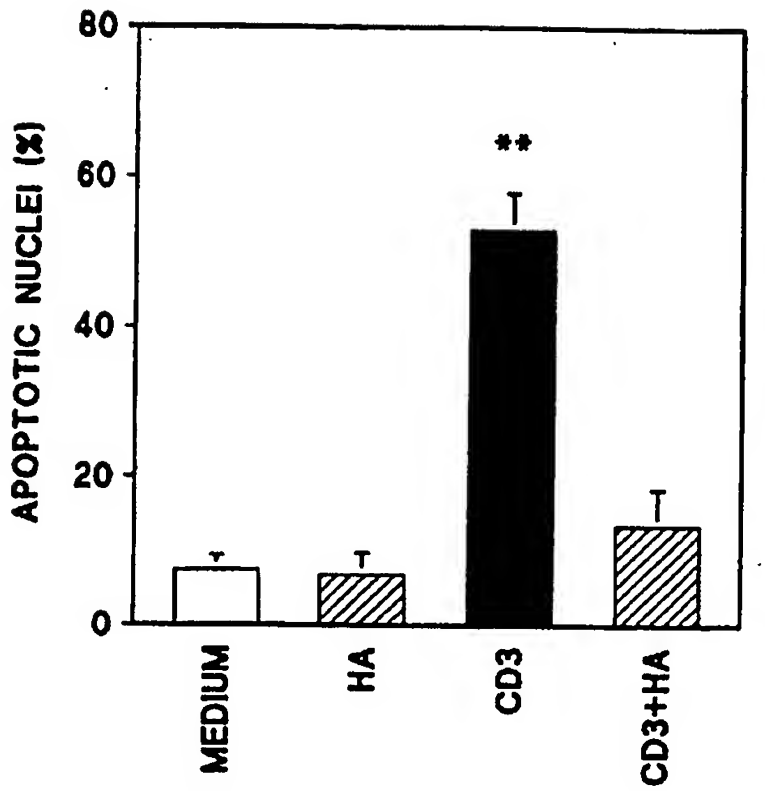

Fig 3. Effect of HA on anti-CD3–induced apoptosis. HA (2 mg/mL) and/or anti-CD3 MoAbs (1 μg/mL) were allowed to adhere in 24-well plates at 4°C as described in Materials and Methods. Cells were cultured for 20 hours on coated plates or in medium alone, then stained with PI, and measured as described above. Data are the means ± SD of three different experiments. $^{**}P < .001$.

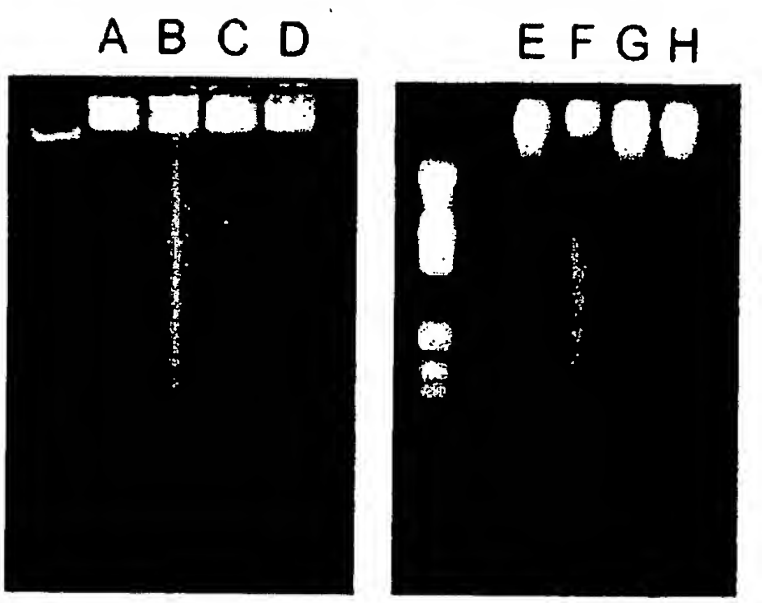

Fig 5. Agarose gel electrophoresis of DNA extracted from cells after 20 hours of in vitro culture in 24-well plates with medium alone (A, E) or coated with anti-CD3 MoAbs (B; 1 μg/mL), anti-CD44 MoAbs (C, G; 1 μg/mL), anti-CD3 plus anti-CD44 (D), DEX (F; 100 nmol/L), or DEX plus anti-CD44 (H; 1 μg/mL).

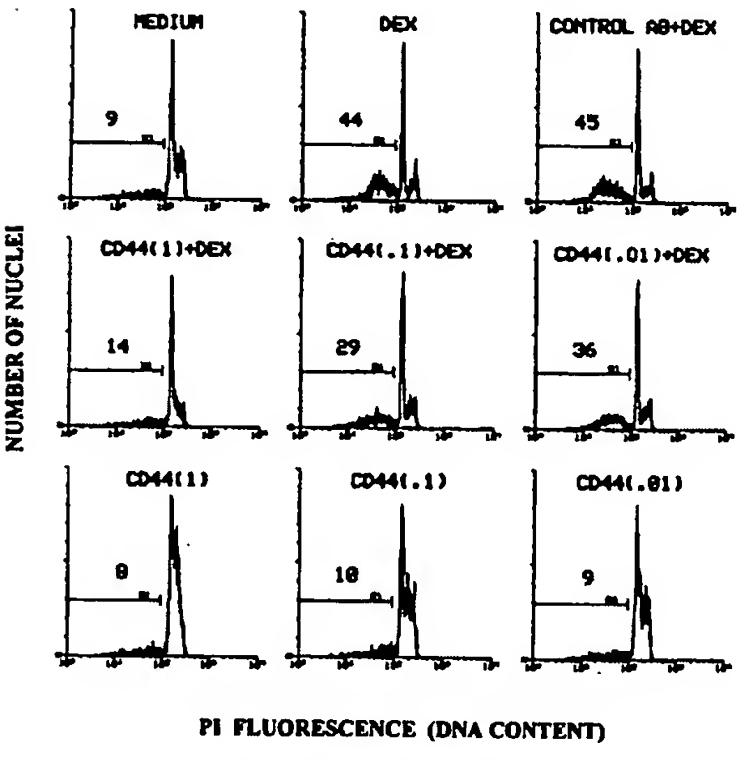

Fig 4. Percentage of apoptotic nuclei of cells treated with DEX alone (100 nmol/L) or DEX plus different concentrations (numbers in parentheses) of anti-CD44 MoAbs adhered on 24-well plates. Analysis of apoptosis by flow cytometry was performed after incubation for 20 hours. The percentage of apoptotic nuclei (broad hypodiploid peak) in a representative experiment is indicated in each histogram.

dried, dissolved in 10 mmol/L Tris-HCl, 1 mmol/L EDTA (pH 8) at 4°C, and the DNA concentration was determined by absorbance at 260 nm with a Perkin-Elmer spectrofluorimeter (Perkin-Elmer Corp, Norwalk, CT). DNA (20 μg) was mixed with 25% Ficoll 400 (Pharmacia, Upsala, Sweden), 0.25% bromophenol blue, and 0.25% xylene cyanol (loading buffer) and then loaded into 1.8% agarose gel wells. The same amount of DNA was loaded in each lane. Electrophoresis was performed in TBE buffer (2 mmol/L EDTA, 89 mmol/L boric acid, 89 mmol/L Tris, pH 8.4), and DNA was visualized by ethidium bromide staining.

PCR. The following reagents were assembled in a final volume of 20 μL: PCR buffer (Perkin Elmer); deoxynucleotide triphosphate (dNTP), 200 μmol/L; specific primers, 0.5 μmol/L each (for bcl-2, forward primer GGAGATCGTGATGAAGTACATAC, reverse primer AGGTATGCACCCAGAGTGATGC); 1 to 2 mmol/L $MgCl_2$; and 2 μL cDNA from reverse transcriptase reaction. After an incubation for 5 minutes at 96°C (hot start) and 2 minutes at the chosen annealing temperature, 0.2 μL Stoffel fragment DNA polymerase (Perkin Elmer) was added. This enzyme was chosen instead of AmpliTaq, because its lack of 5′-3′ exonuclease activity allows a logarithmic increase of PCR products, even during last

Table 1. Anti-CD44 Inhibits the Cell Number Decrease Induced by Treatment With Anti-CD3 or DEX

Treatment	Cell No. ($\times 10^5$/mL)
Medium	6.0 ± 0.8
Anti-CD44	6.7 ± 0.5
Anti-CD3	1.1 ± 0.3
Anti-CD44 + anti-CD3	6.4 ± 0.5*
DEX	1.8 ± 0.4
Anti-CD44 + DEX	5.5 ± 0.6†

Data are means ± SD of three experiments. Cell count was by trypan blue exclusion after incubation for 24 hours.

* $P < .01$ *v* anti-CD3.

† $P < .01$ *v* DEX.

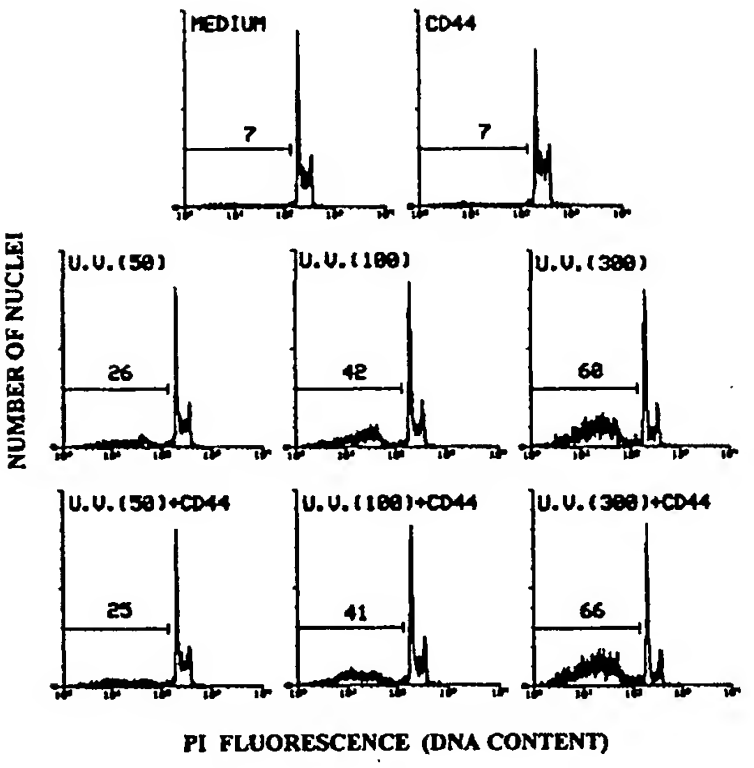

Fig 6. Effect of anti-CD44 MoAbs (24-well plates coated with 1 μg/mL MoAb) on apoptosis induced by different doses of UV (UV J/m² indicated in parentheses) as evaluated by flow cytometric analysis of PI-stained nuclei after 20 hours of in vitro culture. The percentage of apoptotic nuclei (broad hypodiploid peak) in a representative experiment is indicated in each histogram.

cycles. Thirty-five cycles were performed as follows: denaturation at 95°C for 30 seconds, annealing at 61°C for 1 minute, extension at 72°C for 1 minute. The DNA Thermal Cycler 480 (Perkin Elmer) was used. DNA oligonucleotide primers were synthesized in an Oligo-1000 DNA synthesizer (Beckman, Fullerton, CA).

Immunocytologic staining. Staining was performed on cytospin preparations by the alkaline-phosphatase:anti-alkaline-phosphatase (APAAP) procedure using anti-bcl-2 MoAbs (Dako, Glostrup, Denmark) as described previously.[39]

Statistical analysis. Each experiment was performed at least three times. Representative experiments are shown, unless otherwise indicated in the legends. Results, expressed as the mean ± SD of three experiments, are also reported in the text. Because of the non-normal distribution of the data, nonparametric tests (Kruskall-Wallis' analysis of variance) were adopted for statistical evaluation.

RESULTS

Stimulation of CD44 inhibits CD3-mediated activation of apoptosis. Treatment with anti-CD3 MoAbs mimics the effect of Ag-TCR/CD3 interaction. In particular, Ag or anti-CD3 treatment may either activate T-lymphocytes or induce their apoptosis,[14,40] whereas anti-CD44 antibodies augment their CD3-induced activation.[33,34] We used a $CD3^+CD44^+$ T-cell hybridoma subline (3DO; see Materials and Methods) to analyze the effect of CD44 on CD3-induced apoptosis.

Treatment with crosslinked anti-CD3 MoAbs resulted in morphologic changes typical of apoptosis. Analysis of Giemsa-stained cells showed condensation of nuclear chromatin into dense masses and fragmentation of nuclei into apoptotic bodies in some cells (Fig 1). There was a good correlation between the apoptotic cell count by direct microscopic observation and the flow-cytometric evaluation of apoptosis (Fig 2).

The effect of CD44 triggering on anti-CD3–induced apoptosis was then analyzed. The cells were cultured for 20 hours on 24-well plates coated with HA (one of the CD44 ligands) or activating anti-CD44 MoAbs[33,34,34a] in the presence or absence of anti-CD3 MoAbs. Figure 2 shows the results of a representative experiment, obtained by the flow cytometry analysis of PI-labeled nuclei, indicating that treatment with anti-CD44 MoAbs inhibited CD3-induced apoptosis. The mean ± SD of three experiments gave the following results: untreated control, 11% ± 1%; anti-CD3–treated (1 μg/mL), 43% ± 4%; anti-CD3 plus anti-CD44–treated (1 μg/mL), 10% ± 1%; $P < .01$ comparing the anti-CD3–treated and the anti-CD3 plus anti-CD44–treated groups.

The effect of the MoAbs was dose-dependent and specific, as control MoAbs, with the same isotype as anti-CD44 MoAbs, had no effect (Fig 2). Similar results were obtained using thymocytes. The percentage of apoptosis (mean ± SD of three experiments) was 17.0% ± 2% in untreated thymocytes, 30.5% ± 3% in anti-CD3–treated (1 μg/mL), 18.2% ± 2.5% in anti-CD3 plus anti-CD44–treated (1 μg/mL), and 19.0% ± 3% in anti-CD44–treated. The difference was statistically significant ($P < .01$) between anti-CD3–treated and anti-CD3 plus anti-CD44–treated groups.

When soluble anti-CD44 MoAbs were used, no inhibition was detectable. Results of a representative experiment (mean ± SD of two samples per group) gave the following results: untreated 3DO cells, 13.5% ± 2.0%; anti-CD3–treated, 39.8% ± 3.5%; anti-CD3 plus anti-CD44–treated, 41.0% ± 3.0%.

Figure 3 shows the results of experiments (mean ± SD of three experiments) indicating that treatment with HA also inhibited CD3-induced apoptosis. Similar results were obtained when cells were pretreated for 1 hour with anti-CD44 MoAbs or HA and then cultured on anti-CD3–coated plates (not shown).

The results suggest that triggering the CD44 molecule may result in survival of lymphocytes exposed to apoptotic stimuli.

Inhibition of DEX-induced apoptosis. It has been shown that GCH induces apoptosis in both undifferentiated thymocytes and more mature T lymphocytes. Experiments to test the effect of CD44 activation on this induction were performed. Figure 4 gives the flow cytometry profile of nuclei from cells treated with DEX (100 nmol/L) in the presence or absence of crosslinked anti-CD44 MoAbs. The results clearly indicate that stimulation of CD44 countered the DEX-induced apoptotic stimuli. Furthermore, the protective effect of MoAb-treatment was dose dependent and specific, as control MoAbs (at the highest concentration used for anti-CD44 MoAbs), with the same anti-CD44 MoAb isotype, had no effect.

Similar results were obtained when DNA fragmentation, a measure of apoptotic death, was evaluated by the agarose-gel technique. Figure 5 shows that the DNA of anti-CD3– or

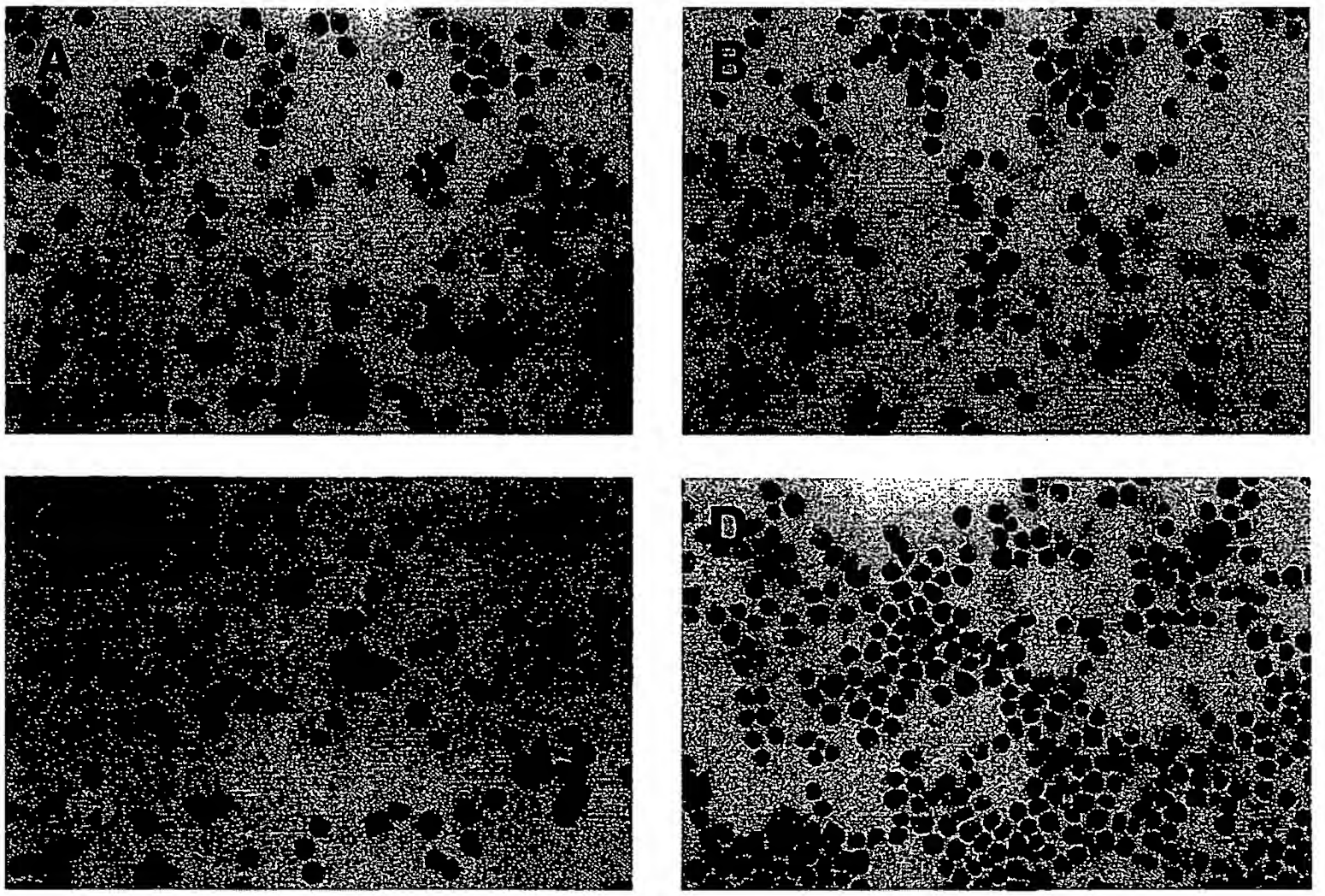

Fig 7. Expression of bcl-2 as evaluated by immunohistologic staining (see Materials and Methods) in cells cultured for 12 hours with medium alone (A), anti-CD44 MoAbs (B), anti-CD3ε MoAbs (C), or anti-CD44 plus anti-CD3ε MoAbs (D). Positive cells show a red staining. MoAbs were adhered as described in Materials and Methods.

DEX–treated cells was degraded with the oligonucleosomal ladder pattern of apoptosis, whereas the DNA fragmentation of cells incubated with anti-CD44 MoAbs alone was comparable with that of medium-treated controls. Treatment with anti-CD44 MoAbs strongly countered fragmentation induced by anti-CD3 MoAbs (Fig 5, left) or DEX (Fig 5, right).

Cell count analysis by trypan blue exclusion assay confirmed the protective effect of CD44 triggering. Data reported in Table 1 indicate that treatment with anti-CD44 MoAbs countered the cell number decrease induced by DEX or anti-CD3 MoAbs.

These results confirm that stimulation of CD44 can protect cells from CD3- and DEX-induced DNA fragmentation.

UV-induced apoptosis is not inhibited by CD44 stimulation. UV-treatment induces DNA fragmentation and apoptotic death by a p53-dependent mechanism.[35,41,42] We tested the effect of anti-CD44 MoAbs on apoptosis of cells exposed to different UV doses. Figure 6 shows that stimulation of

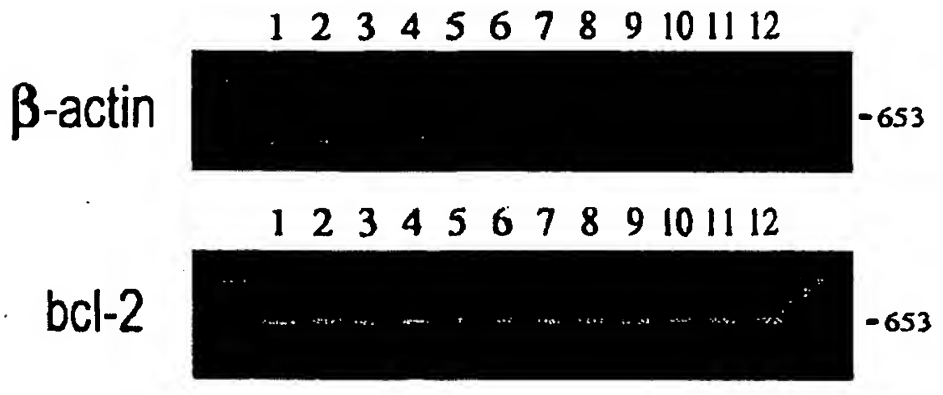

Fig 8. Expression of bcl-2 as evaluated by RT-PCR (as described in Materials and Methods) in cells cultured for 1 hour (lanes 1 through 6) or 4 hours (lanes 7 through 12) with medium alone (lanes 1 and 7), anti-CD44 (1 μg/mL) MoAbs alone (lanes 2 and 8), DEX (100 nmol/L) alone (lanes 3 and 9), anti-CD3ε (1 μg/mL) MoAbs alone (lanes 4 and 10), DEX plus anti-CD44 (lanes 5 and 11), or anti-CD3 plus anti-CD44 (lanes 6 and 12).

CD44 did not counteract UV-induced cell death. Similar results were obtained when cells were treated with HA (not shown).

CD44 does not induce bcl-2 expression. The mechanisms responsible for this antiapoptotic effect were investigated by analyzing the role of bcl-2. Treatment with anti-CD44 MoAbs alone or in combination with DEX or anti-CD3 MoAbs did not produce any change in bcl-2 expression as evaluated by either immunocytochemistry (Fig 7) or by PCR (Fig 8).

DISCUSSION

We have studied the role of CD44 in modulation of apoptosis. Apoptotic death occurs during embryogenesis and normal development and is activated by nontoxic stimuli.[1,3-6] T lymphocytes are particularly sensitive to apoptosis caused either by activation through Ag-TCR interaction or treatment with anti-CD3 MoAbs.[13,14] However, several other factors can modulate apoptotic death during lymphocyte development. ILs and GCH contribute to the effects of CD3/TCR complex activation, resulting in expansion or deletion of thymocytes or more differentiated peripheral T lymphocytes.[17-20]

Mutual exclusion between two apoptosis inducers, anti-CD3 MoAbs and DEX, has also been described in thymocytes.[43] We have recently shown that two apoptotic treatments, heat shock and DEX, inhibit each other, suggesting that apoptotic agents can counteract apoptosis when used in combination,[43,44] and that a complex of stimuli could be involved in modulating cell death.

Further signals may be involved in regulating the Ag-TCR/CD3–driven apoptosis of T lymphocytes, including those activated by adhesion receptors. Recent observations have shown that integrin-mediated signals play a relevant role in the control of cell survival.[21,22] Epithelial and endometrial cells undergo apoptosis when they lose contact with their underlying matrix, indicating that integrin signals may control the apoptotic response.[22]

Because CD44, which acts as a major receptor for HA, is involved in the homing, adhesion, and activation of T lymphocytes,[27,31] we performed experiments to analyze the influence of CD44 stimulation on lymphocyte apoptosis using a susceptible CD3+CD44+ cell line (Figs 1 through 5). This is a useful model for studying T-cell responses and functions at the single-cell level and allows problems due to heterogeneity of freshly isolated T lymphocytes to be circumvented.

Our data indicate that CD44 stimulation by HA or anti-CD44 MoAbs resulted in inhibition of apoptosis induced by anti-CD3 MoAbs or treatment with DEX. The protective effect was evident when analyzed by flow cytometric analysis (Figs 2 through 4), by the agarose gel assay (Fig 5), which revealed inhibition of DNA fragmentation, and by cell count (Table 1). Furthermore, the inhibiting effect of anti-CD44 MoAbs was dose-dependent and specific, as it was not induced by control MoAbs (Fig 2).

When apoptosis was induced by UV (Fig 6), a p53-dependent apoptotic pathway,[35,41,42] anti-CD44 MoAbs did not inhibit cell death, suggesting that CD44 may use a specific pathway to inhibit apoptosis induced by activation of the CD3/TCR complex or by GCH, but not by other inducing systems. Because protection against apoptosis supported by IL-3 and IL-4 seems to be related to an upregulation of bcl-2,[45] we investigated whether the signals induced by CD44 molecules produced overexpression of bcl-2 oncoproteins or specific mRNA. Engagement of the CD44 molecule by either anti-CD44 MoAb did not produce any appreciable increase in bcl-2 levels compared with untreated controls (Figs 7 and 8).

Although the in vivo importance of our results remains to be shown, the data suggest that CD44 is not only physiologically involved in the control of lymphoid cell homing and activation, but also modulates lymphocyte apoptosis. It has been recently reported that during peripheral T lymphocyte depletion by apoptosis after CD4 ligation, CD44− T cells are selectively eliminated, whereas CD44+ memory T cells are activated.[46] One possible explanation is that the CD44 contribution to T-cell selection is a survival signal capable of modulating the activating and/or deleting signals induced by other membrane molecules, including the CD3/TCR complex. This antiapoptotic activity could be important in terms of immunologic memory by contributing to survival of peripheral immune T cells.

In conclusion, our results suggest that CD44 may exert immunoregulatory effects by inhibiting T-cell apoptosis induced by CD3/TCR activation or treatment with GCH, and confirm that a number of agents may be involved in maintaining T-cell survival during development. Studies are in progress to examine the intracellular signals activated by CD44 stimulation that may be involved in modulating lymphocyte survival.

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Evidence for in vivo production of Humanin peptide, a neuroprotective factor against Alzheimer's disease-related insults

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Abstract

An unbiased functional screening with brain cDNA library from an Alzheimer's disease (AD) brain identified a novel 24-residue peptide Humanin (HN), which suppresses AD-related neurotoxicity. As the 1567-base cDNA containing the open reading frame (ORF) of HN is 99% identical to mitochondrial 16S ribosomal RNA as well as registered human mRNA, it was elusive whether HN is produced in vivo. Here, we raised anti-HN antibody and found that long cDNAs containing the ORF of HN (HN-ORF) produced the HN peptide in mammalian cells, dependent on the presence of full-length HN-ORF. Immunoblot analysis detected a 3-kDa protein with HN immunoreactivity in the testis and the colon in 3-week-old mice and in the testis in 12-week-old mice. HN immunoreactivity was also detected in an AD brain, but little in normal brains. This study suggests that HN peptide could be produced in vivo, and would provide a novel insight into the pathophysiology of AD.

Keywords: Humanin; Alzheimer's disease; In vivo expression; Humanin mRNA; Humanin peptide; Mitochondrial 16S ribosomal RNA with a polyA tail; Neuronal death; Rescue factor

To find molecules inhibiting Alzheimer's disease (AD)-relevant neurotoxicity, we applied 'death-trap' screening [2] to V642I-mutated amyloid precursor protein-inducible neuronal cells [8] with an expression cDNA library from an occipital lobe of an autopsy-diagnosed AD brain. With this screening, we identified the open reading frame (ORF) of Humanin (HN) cDNA, encoding a novel 24-residue polypeptide, which suppresses neurotoxicity by various AD genes and Aβ peptides without effect on neurotoxicity by long polyglutamine, superoxide dismutase-1 mutants, or prion peptide [3–5]. The neuroprotective action of HN-ORF cDNA was mediated by the extracellularly secreted peptide encoded by the HN-ORF (HN peptide). Synthetic HN (sHN) peptide reproduced the neuroprotection by HN-ORF cDNA at concentrations as low as that of secreted HN peptide in the culture medium. Therefore, the HN-ORF exerts neuroprotection against AD-relevant insults via expression and secretion of the corresponding HN peptide.

The obtained long HN cDNA (1567 bases including a polyA tail; #AY029066) containing HN-ORF is 99% identical to positions 1680–3231 of mitochondrial DNA (mtDNA; #AF347013; 1679–3230 in the case of #AB055387), corresponding to 16S rRNA. Therefore, one possibility is that HN peptide is an artificial protein encoded by a non-functional ORF in 16S rRNA. In this case, HN-ORF-containing polyA$^+$RNA only represents 16S rRNA attaching a polyA tail. All mitochondrial rRNA transiently attach a polyA tail during transcription [1]. Consistent with this possibility, the long 1567-base HN cDNA has an unusual structure in which it encodes the short HN-ORF of 75 bases located 950 bases downstream of the 5′ end of the cDNA, and in which at least seven potential in-frame ORFs, each with a termination codon, are included in the 5′

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EXHIBIT 3

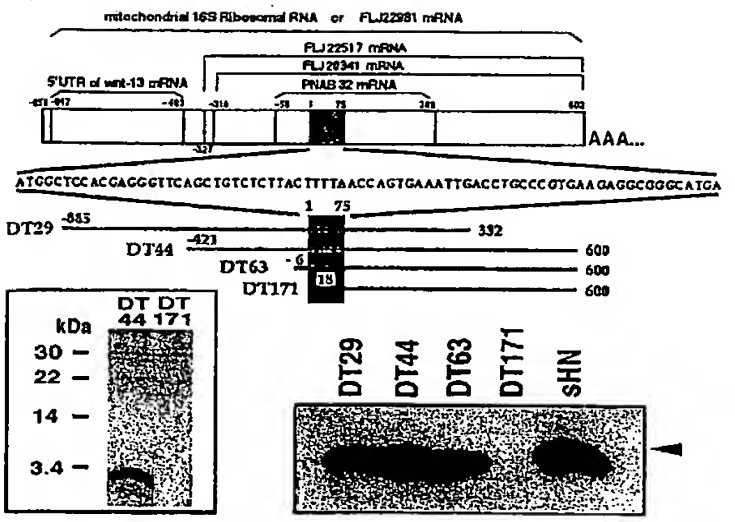

Fig. 1. Expression of the long HN cDNA inserted into pEF-BOS. F11 cells (7×10^4 cells/well in a six-well plate) were transfected with various DT clones by lipofection (1 μg DT clone, 2 μl LipofectAMINE, 4 μl PLUS reagent (GibcoBRL)). Seventy-two hours after lipofection, cultured media (lower right panel) and cell lysates (lower left panel) were analyzed with P04. The cultured medium was added with a protease inhibitor cocktail (PIC; Roche Diagnostics), and the sample (20 μl/lane) was submitted to Tris–Tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; 16% T, 3% C), P04 (1:3000 dilution). For analysis of cell lysates, cells were washed twice with phosphate-buffered saline, and suspended in 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1% Triton X-100, and PIC. After freezing and thawing twice, the cell lysate was centrifuged at 15,000 rpm for 10 min at 4 °C and the supernatant (20 μg/lane) was submitted to Tris–Tricine PAGE. The arrow indicates 3.4 kDa. The No. 1 base corresponds to the first base of the HN-ORF (shaded area), one base before which is numbered −1. DT clones were in pEF-BOS, an elongation-factor-driven plasmid.

region of the HN-ORF. Although HN peptide has no signal peptide sequence at the N terminus, it has been clarified that secretory activity is encoded by the full-length HN per se [3]. On the other hand, regions 92–95% homologous to the long HN cDNA over 1000 bases exist on human chromosomes 5, 11, and X [3]. Thus, another possibility is that the source of HN cDNA is nuclear and that HN peptide is natively produced from HN mRNA. Indeed, long regions in the HN cDNA containing HN-ORF are 99% identical to certain registered human mRNAs (Fig. 1). In this case, as all cells more or less contain polyA$^+$16S rRNA, HN-ORF-hybridizable polyA$^+$RNA is a mixture of real HN mRNA and polyA$^+$16S rRNA. The last possibility is that mitochondrial 16S rRNA with a polyA tail produces HN peptide. It is therefore important to determine whether HN peptide is produced in certain tissues in vivo.

We raised a polyclonal antibody against the HN peptide, termed P04, and examined whether expression of long HN cDNAs obtained by the 'death-trap' screening (DT clones) causes secretion of HN peptide in transfected F11 neuronal hybrid cells [3–5] (Fig. 1). Transfection with 1217-base DT29, 1021-base DT44, 606-base DT63—each of which contain HN-ORF—resulted in the expression and secretion of HN peptide. In contrast, transfection with 583-base DT171—which does not include the initiation codon—did not do so. Hence, transcriptional activation of the long HN cDNA could lead to the production of HN peptide in mammalian cells, despite its unusual structure for peptide production.

Using P04, which was raised against human HN (hHN), we next examined tissue expression of the HN peptide in mice, although before this study, there had been no information of whether P04 interacts with mouse HN (mHN). We found the antigenic peptide at around 3 kDa, equivalent to the estimated molecular weight (MW) of hHN peptide, in the testis and the colon of 3-week-old mice (Fig. 2A). It should be noted that no information, including its MW, was available about the mHN peptide. However, these

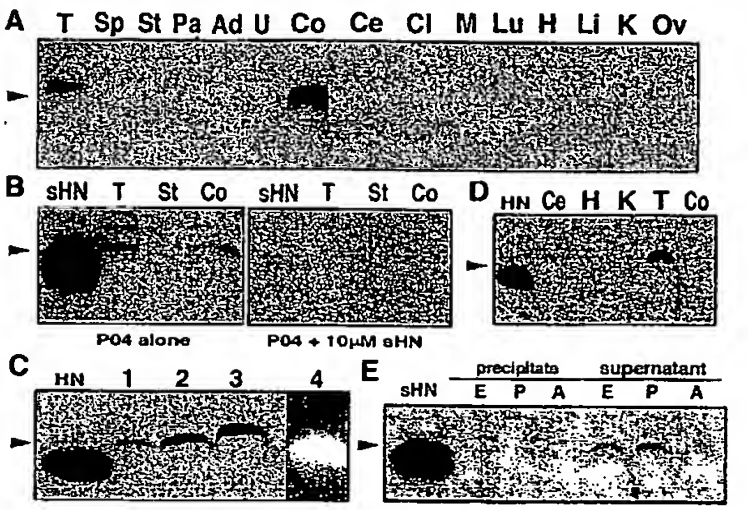

Fig. 2. Tissue expression of the 3-kDa peptide with HN immunoreactivity. (A) Lysates (50 μg/lane) of tissues obtained from 3-week-old mice (T, testis; Sp, spleen; St, stomach; Pa, pancreas; Ad, adrenal gland; U, uterus; Co, colon; Ce, cerebrum; Cl, cerebellum; M, medulla oblongata; Lu, lung; H, heart; Li, liver; K, kidney; Ov, ovary) were submitted to immunoblot analysis with P04 (1:5000 dilution). The 3-kDa band in the testis of 3-week-old mice was observed 22 times in 23 similarly performed experiments using the testes pooled from 10–15 individual mice. The band in the colon of 3-week-old mice was observed eight times in nine experiments. The arrows indicate 3.4 kDa. Tissue sample were lysed by dilution buffer (25 mM Tris–HCl (pH 7.5), 10 mM EDTA, and PIC) plus 1% Triton X-100. After centrifugation at 15,000 rpm, protein concentration of the supernatant was measured and the equivalent volume of dilution buffer was added to set Triton X-100 at 0.5%. (B) Tissue lysates of 3-week-old mice were immunoblotted by P04 with (left panel) or without (right panel) preabsorption with 10 μM sHN. (C) Increasing amounts (#1, 25 μg; #2, 50 μg; #3, 100 μg) of the same testis sample from 3-week-old mice were analyzed with P04. In #4, the testis sample was submitted to Tris–Tricine PAGE and immersed in 0.005% (w/v) Rhodamine 6G solution for 30 min. (D) Tissue lysates of 12-week-old mice were analyzed with P04. (E) The organic solvent (precooled at −20 °C; E, ethanol; P, 2-propanol; A, acetone; finally 80% (v/v)) was added to the lysates of the testes obtained from 3-week-old mice on ice. The sample rotated for 30 min at 4 °C was then centrifuged at 15,000 rpm for 10 min at 4 °C. The supernatant was evaporated. The sample buffer (62.5 mM Tris–HCl (pH 6.8) 2% sodium dodecyl sulfate, 10% glycerol, 0.01% phenol red) was added to the precipitate or the supernatant, followed by denaturation at 95 °C for 10 min.

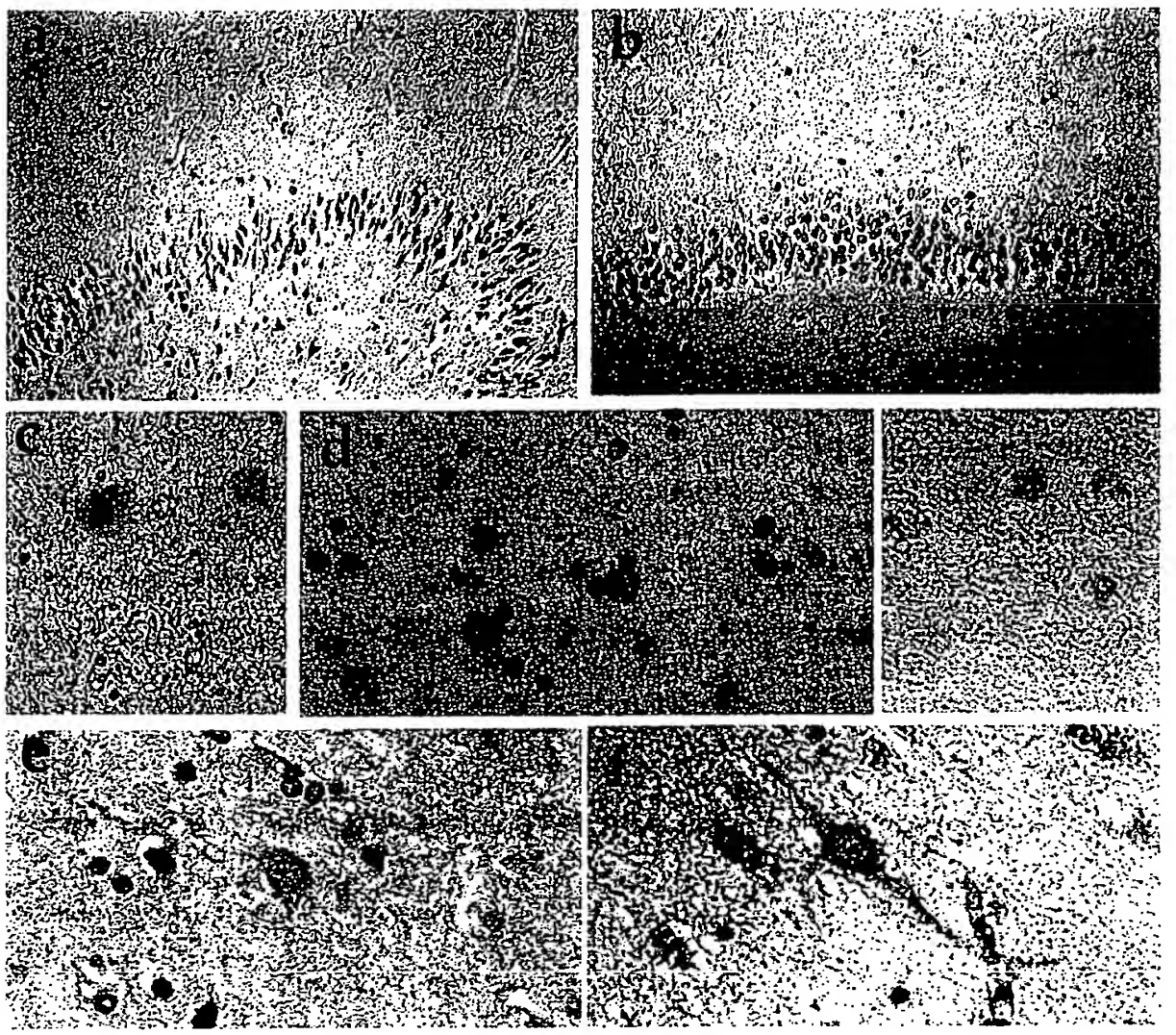

Fig. 3. Immunohistochemical analysis of an autopsy-diagnosed AD brain. The hippocampal sections of an age-matched control brain (a) or an AD brain (b) were immunostained with P04. The view in (a) focuses on the area where P04-stained cells were mostly observed. Senile plaques in the different sections from the same hippocampus of the AD brain were stained brown by anti-Aβ antibody (MBL, Nagoya, Japan) in (c). In (d), the magnified views of hippocampal sections stained with P04 (left panel), shown in (b), or preimmune serum (right panel) are indicated. In (e) and (f), P04-stained neurons in the occipital lobe of the age-matched control brain (e) or the AD brain (f) are shown. All these samples were counterstained with methylgreen (a–d,f) or HE staining (e). The brain sections were obtained from an autopsy-diagnosed AD patient, who had been clinically cared for by Saiseikai Central Hospital (Mita, Tokyo, Japan).

bands disappeared when P04 preadsorbed with sHN was used (Fig. 2B; preadsorption by 10 μM sHN for 30 min at room temperature). Although these bands in the testis and the colon were observed at a MW a little higher than that of sHN, it was observed that the position of the 3-kDa band shifted upward when the amount of each loaded sample was increased (Fig. 2C, lanes #1–#3). Tissue samples contained a hydrophobic material at around 3 kDa (Fig. 2C, lane #4), which upwardly shifted the position of the 3-kDa HN antigenic band. As it was stained whitish yellow by Rhodamine 6G, this hydrophobic material consisted of neutral lipid. As compared with that in the testis, the contained hydrophobic material appeared to be less in the colon, resulting in less upward shift of the HN antigenic band. With inappropriate concentrations of Triton X-100, the HN antigenic band appeared to be covered by the lipid area, resulting in little staining of the band. To visualize the HN antigenic band, it was necessary to lower the concentration of Triton X-100 in the tissue samples to 0.5%. In 12-week-old mice, this band was only observed in the testis and was not detected in other tissues, including the colon (Fig. 2D). These data indicate that the 3-kDa peptide with HN immunoreactivity is produced in vivo in a tissue- and age-dependent manner.

We attempted to specify the MW of the HN-immunoreactive band by delipidating the testis samples by organic solvent extraction, and found that the 3-kDa HN-immunoreactive protein moved to organic solvent extracts (Fig. 2E). As sHN dissolved with bovine serum albumin in water did not move to organic solvent extracts (not shown), the 3-kDa HN-immunoreactive protein would be associated with lipids in the tissue samples. Therefore, mHN would have a size similar to that of hHN, but with its gel position upwardly shifted by co-migrated lipids. However, we could not exclude the possibility that the MW of mHN is a little higher than that of hHN or that mHN in tissues is posttranslationally modified.

We next examined whether HN immunoreactivity is present in a human AD brain, as we obtained HN cDNA from the cDNA library constructed from an AD occipital lobe [3]. In an AD brain, HN immunoreactivity was detected

in some of the intact large neurons in the occipital lobes (Fig. 3f). There was no similar immunostaining in neurons in an occipital lobe in an age-matched control brain (Fig. 3e). In the AD brain, HN immunoreactivity was also detected in small, round reactive glias (Fig. 3d, left panel). This type of immunoreactivity was widely distributed in the AD brain, most abundantly in the hippocampus. The age-matched control brain exhibited only few HN-immunoreactive glias (Fig. 3a). There was no apparent geographic relationship between these small round glias and senile plaques (Fig. 3c). Preimmune serum stained none of these cells (Fig. 3d).

We have herein provided evidence that HN peptide could be produced in vivo. This study suggests that several normal tissues in mice and certain cells in a human AD brain produce HN peptide. As the HN peptide appeared to be associated with lipids in tissue samples, we must consider the influence of tissue-, age-, or disease-dependent alterations in lipid composition on the positive immunoreactivity of the tissue HN peptide, which might be relevant to the limited number of positive tissues or cells. It remains unclear whether the DNA source of the tissue HN peptide is mitochondrial or nuclear. Although it may be an unusual idea that HN peptide is produced from mitochondrial 16S rRNA, we cannot exclude this possibility, because long HN cDNA, virtually identical to polyA$^+$16S rRNA, produced HN peptide when driven by the elongation factor promoter. Harai et al. [7] found that AD neurons associate with mitochondrial abnormalities. It is therefore intriguing to pursue the possibility that HN is produced from mitochondrial 16S rRNA and that its altered production contributes to neuronal damages in AD.

However, there are pieces of evidence against this possibility. Among others, the tissue distribution of HN-ORF-hybridizable polyA$^+$RNA [3] was quite discrepant from that of the HN-immunoreactive peptide. If all mitochondrial polyA$^+$16S rRNA lead to the translation of HN peptide, the peptide distribution would reflect the polyA$^+$RNA distribution at least to some extent. However, there remains a possibility that the stability of HN-ORF-hybridizable polyA$^+$RNA is drastically different from that of HN peptide in a tissue-dependent manner.

To further clarify this issue, we examined ρ^0 HeLa cells, which lack mtDNA [6]. Ordinary HeLa cells abundantly contained HN-ORF-hybridizable total RNA, whose major band was identical in size to that of mitochondrial 16S rRNA (Fig. 4A). In contrast, ρ^0 cells contained little HN-ORF-hybridizable total RNA, which was confirmed by reverse transcription-polymerase chain reaction (RT-PCR; Fig. 4B). Virtually all HN-ORF-hybridizable RNA would thus represent mitochondrial 16S rRNA in HeLa cells. Accordingly, neither HeLa cells nor ρ^0 cells produced the 3-kDa peptide with HN immunoreactivity (Fig. 4C). Thus, mitochondrial polyA$^+$16S rRNA may not contribute to the production of the HN peptide. However, to obtain direct evidence, similar experiments using cells producing HN peptide are necessary.

Even if mitochondrial 16S rRNA does not encode HN peptide, there is another possibility that 16S rRNA critically interferes the expression of HN peptide. As reported by Hirai et al. [7], a striking increase of mtDNA occurs in the cytoplasm of damaged AD neurons. If so, the antisense strand of the cytoplasmic mtDNA of 16S rRNA could inhibit HN expression, as the sequence of 16S rRNA is virtually identical to that of HN cDNA. Whether HN peptide is present or not, HN peptide and its derivatives provide a novel insight into the development of clinical AD therapies. However, this study suggests that HN peptide could be produced in vivo and that HN-ORF-hybridizable polyA$^+$RNA may consist of two components, HN mRNA and mitochondrial polyA$^+$16S rRNA. This study provides a novel insight into physiological neuroprotection as well as the pathophysiology of AD.

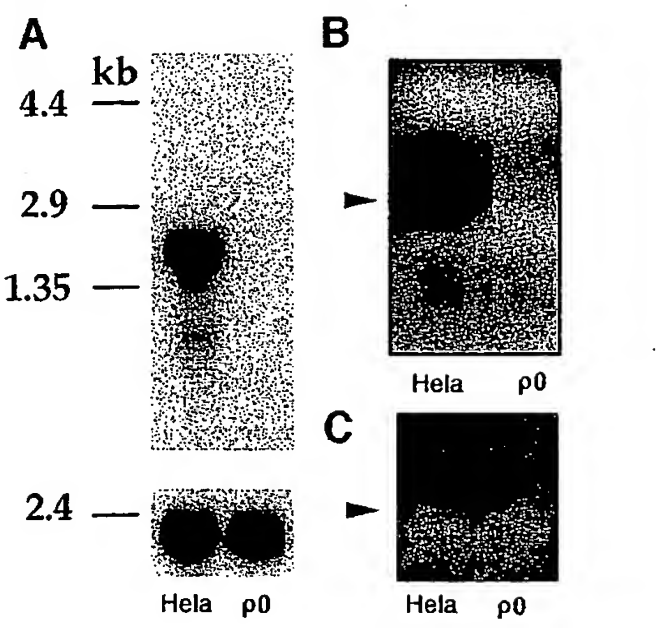

Fig. 4. HN-ORF-hybridizable RNA in mtDNA-lacking HeLa cells. (A) Total RNA obtained from HeLa cells or ρ^0 HeLa cells was submitted to Northern blot analysis with antisense HN-ORF (upper panel) or a β-actin probe (bottom panel). (B) RT-PCR of the HN cDNA fragment (−574 to +100; arrow) was performed with cDNA from HeLa cells or ρ^0 cells by 5′-CCACAGAACCCTC-TAAATCC-3′ and 5′-CATAGGGTCTTCTCGTCTTG-3′. RNA was treated with DNase I before RT reaction. (C) Lysates of HeLa cells or ρ^0 cells were analyzed with P04. The membrane was overexposed so that the hydrophobic area around 3 kDa became visible.

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